BREAST CANCER RESISTANCE, BIOMARKERS AND THERAPEUTICS DEVELOPMENT IN THE ERA OF ARTIFICIAL INTELLIGENCE

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PUBLISHED IN: Frontiers in Molecular Biosciences

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-83250-477-2
DOI 10.3389/978-2-83250-477-2

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BREAST CANCER RESISTANCE, BIOMARKERS AND THERAPEUTICS DEVELOPMENT IN THE ERA OF ARTIFICIAL INTELLIGENCE

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Citation: Ali, L., Khan, A., Wei, D., eds. (2022). Breast Cancer Resistance, Biomarkers and Therapeutics Development in the Era of Artificial Intelligence. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83250-477-2

Table of Contents

frontiers | Frontiers in Molecular Biosciences

TYPE Editorial
PUBLISHED 28 September 2022
DOI 10.3389/fmolb.2022.1034990

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Molecular Diagnostics and Therapeutics, a section of the journal Frontiers in Molecular Biosciences

RECEIVED 02 September 2022
ACCEPTED 12 September 2022
PUBLISHED 28 September 2022

CITATION
Khan A, Ali L and Wei D-Q (2022), Editorial: Breast cancer resistance, biomarkers and therapeutics development in the era of artificial intelligence.
Front. Mol. Biosci. 9:1034990.
doi: 10.3389/fmolb.2022.1034990

Editorial: Breast cancer resistance, biomarkers and therapeutics development in the era of artificial intelligence

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KEYWORDS

breast cancer, artificial intelligence, biomarkers, daignosis, therapeutic

Editorial on the Research Topic
Breast cancer resistance, biomarkers and therapeutics development in the era of artificial intelligence

Breast cancer is globally the most diagnosed form of cancer in females, and one of the major causes of death from cancer (~2.3 million cases according to Sung et al (2021) Global cancer statistics, 2020) (Sung et al., 2021). Breast cancer is a clinical condition with distinct molecular features and genetic profiles composed of various subtypes. Triple Negative Breast Cancers are one of the most aggressive breast cancers and, particularly in comparison to other Breast Cancers, have a higher 5-years death rate after treatment. Breast cancers in young females appear to be detected at more advanced stages and exhibit more aggressive biological features compared to tumors that arise in older patients. Additionally, various factors determine the emergence of drug resistance in multifactorial diseases like breast cancer. In this regard, computational methods and the recent machine learning algorithms had exponentially increased the research against different diseases and biological processes. From virtual drug screening to the molecular mechanism and from vaccine designing to therapeutic platform development, computational approaches are of great interest. They have significantly accelerated the comprehension of genomics patterns, proteomics, structure determination, mutation stability, function correlation, and also tracing. However, our knowledge about complicated breast cancer is very limited. For instance, these methods have been previously used to understand the mechanism of drug resistance in breast cancer and discover novel biomarkers for effective treatment (Khan et al.; Khan et al., 2020b). Understanding BC mechanisms is essential to determine how the mutations help to survive by protecting themselves from the hosts' immune defense. Computational approaches in predicting the impact of these mutations on the protein structure, function and binding to other

partners offer great promise for devising therapeutic strategies. No effective and final therapeutic strategies are available. Hence, further research is needed to increase our understanding and forestall this disease. Therefore, the current study issue aims to focus on the recent advances in the development of novel biomarkers, mutation identification, and therapeutics against BC. In this special issue, 11 articles including original research articles and review articles are published with a focus on breast cancer using state-of-the-art computational methods.

In the very first study, large-scale mutational analyses have been performed to systematically screen the most damaging mutation in the recently characterized protein in breast cancer known as Pirin. Suleman et al. reported V257A, I28T, and I264S mutations as important in the context of functional variation caused by them. Using molecular simulation methods to capture the behavior of these mutations in contrast to the wild type at atomic level. They revealed a greater stability drift in the structure of PIR mediated by these mutations (Suleman et al.). Similarly, Muneer et al. used virtual drugs screening approaches to discover novel drugs against the breast cancer biomarker. Using the MPD3 database, drugs were screened against VISTA protein and validated by using molecular simulation-based approaches. From this study Three compounds, Paratocarpin K (PubChem ID: 14187087), 3-(1H-Indol-3-yl)-2-(trimethylazaniumyl)propanoate (PubChem ID: 3861164), and 2-[(5-Benzyl-4-ethyl-1,2,4-triazol-3-yl)sulfanylmethyl]-5-methyl-1,3,4-oxadiazole (PubChem ID: 6494266), having binding energies stronger than −6 kcal/mol were found to have two common hydrogen bond interactions with VISTA active site residues: Arg54 and Arg127. The dynamics of the compound–VISTA complexes were further explored to infer the binding stability of the systems. These hits can be used *in vitro* and clinical studies to determine their efficacy and usage against the BC (Muneer et al.).

In another study using the microenvironment clustering method, relapse-free survival (RFS) of different phenotypes in 100 patients with RNA sequencing-based expression data from the PATTERN trial were compared (Zhu et al.). According to this study, the microenvironment phenotypes in TNBC may be able to predict both the node-positive patients' prognosis and the outcome of high-risk node-negative patients. Likewise, the tumor microenvironment (TME) model was proposed as the best approach for biomarker identification (Xu et al.). The other reports further demonstrated the role of single or multiple viruses in the initiation and progression of breast cancer. The review article stressed on constraining the viral-based BC spread by applying effective infectious measures (Afzal et al.). Furthermore, novel biomarkers identification and their role in the detection and progression of BC have been revealed. The review reported different biomarkers based on the type i.e. nucleic acids, proteins, or other groups. The two approaches i.e., DNA methylation and miRNA profiling were defined as the best approaches for the accurate detection of BC (Almansour et al.; Afzal et al.). In addition, the role of Frizzleds (FZDs), human receptors, has been reported in BC patients. TargetScan and miRabel target-prediction databases were used to identify the potential microRNA that regulates the expression of FZD. FZD6 was particularly identified as highly expressed among the others in BC samples. The study revealed the role of FZD6-mediated signaling of molecular silencing machinery of the Wnt pathway (Assidi et al.). Moreover, Rui et al. identified Mir-4728 as an essential biomarker for the diagnosis and prognosis of HER2-positive BC while the role of CC-chemokine receptor 7 (CCR7) and its potential druggability was explored (Alrumaihi et al.; Rui et al.). Finally, Almansour et al. explored the essential therapeutic approaches including the earlier and contemporary approaches for the treatment of BC. The review provides the choice of treatment options based on the omics technologies. The authors stressed on personalized treatment as an effective approach for better clinical outcome (Mehmood et al.). In sum, this collection piled up studies that provide a deep understanding of drug resistance, biomarkers development, diagnosis, and treatment options for the efficient management of BC. This special issue piled up essential information regarding BC drug resistance and therapeutics. This issue provides essential understanding of the key events that are responsible drug resistance, cancer progression and treament. This information can be used for structure-based and rationale based therapeutics development to control BC.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication. All authors were involved in writing the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers in Molecular Biosciences

ORIGINAL RESEARCH
published: 28 June 2021
doi: 10.3389/fmolb.2021.692835

Mutational Landscape of Pirin and Elucidation of the Impact of Most Detrimental Missense Variants That Accelerate the Breast Cancer Pathways: A Computational Modelling Study

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OPEN ACCESS

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Specialty section:
This article was submitted to Molecular Diagnostics and Therapeutics, a section of the journal Frontiers in Molecular Biosciences

Received: *09 April 2021*
Accepted: *07 June 2021*
Published: *28 June 2021*

Citation:
Suleman M, Tahir ul Qamar M, Saleem S, Ahmad S, Ali SS, Khan H, Akbar F, Khan W, Alblihy A, Alrumaihi F, Waseem M and Allemailem KS (2021) Mutational Landscape of Pirin and Elucidation of the Impact of Most Detrimental Missense Variants That Accelerate the Breast Cancer Pathways: A Computational Modelling Study. Front. Mol. Biosci. 8:692835. doi: 10.3389/fmolb.2021.692835

Pirin (*PIR*) protein is highly conserved in both prokaryotic and eukaryotic organisms. Recently, it has been identified that *PIR* positively regulates breast cancer cell proliferation, xenograft tumor formation, and metastasis, through an enforced transition of G1/S phase of the cell cycle by upregulation of E2F1 expression at the transcriptional level. Keeping in view the importance of *PIR* in many crucial cellular processes in humans, we used a variety of computational tools to identify non-synonymous single-nucleotide polymorphisms (SNPs) in the *PIR* gene that are highly deleterious for the structure and function of *PIR* protein. Out of 173 SNPs identified in the protein, 119 are non-synonymous, and by consensus, 24 mutations were confirmed to be deleterious in nature. Mutations such as V257A, I28T, and I264S were unveiled as highly destabilizing due to a significant stability fold change on the protein structure. This observation was further established through molecular dynamics (MD) simulation that demonstrated the role of the mutation in protein structure destability and affecting its internal dynamics. The findings of this study are believed to open doors to investigate the biological relevance of the mutations and drugability potential of the protein.

Keywords: nsSNPs, PIR protein, breast cancer, MD simulations, PIR

INTRODUCTION

Pirin (*PIR*) protein is considered highly conserved in both prokaryotic and eukaryotic organisms; however, its biological functions are poorly described (Dunwell et al., 2001; Pang et al., 2004). Pirin is reported as a biomarker in breast cancer, which is abnormal and irregular proliferation of cells associated with inappropriate stimulation of pathways involved in signal transduction (Feitelson

et al., 2015; Riaz et al., 2017; Chang et al., 2019). The crystal structure of the human *PIR* gene revealed its quercetinase (acts on quercetin flavonoid) and regulatory functions in many cellular pathways like an inhibitor of protein kinase, antioxidant as well as putative transcriptional co-factor (Chen et al., 2004; Wendler et al., 1997). Previous studies reported the overexpression of *PIR* in different neoplastic transformation and its role in the enhancement of tumor formation due to inducing the expression of Bcl3 by forming the ternary complex with proto-oncogenes Bcl3 and NF-kB (Zhu et al., 2003; Massoumi et al., 2009). Recently, it has been identified that *PIR* positively regulates breast cancer cell proliferation, xenograft tumor formation, and metastasis, through an enforced transition of G1/S phase of the cell cycle by upregulation of E2F1 expression at the transcriptional level (Suleman et al., 2019). It was a significant breakthrough in unveiling the hidden function of *PIR* in the field of cancer.

The most frequently occurring genetic variations are single-nucleotide polymorphisms (SNPs), which disturb both coding and non-coding regions of DNA. SNPs occur in every 200–300 bp in the human genome and consist of about 90% of the total genetic variations in the human genome. The nsSNPs (non-synonymous single-nucleotide polymorphisms) are the various mutations that occur in exonic regions and change the protein sequence, structure, and normal function by triggering modifications in the mechanism of transcription and translation.

Recently, various *in silico* computational tools, methods, and approaches were adopted to investigate the possible role of non-synonymous variation in protein structure and function efficiently and accurately (Kumar et al., 2009; Wadood et al., 2017; Muneer et al., 2019). These methods are of great interest to decipher important molecular mechanisms from protein–protein binding to drug development (Khan et al., 2020a; Khan et al., 2020b; Khan et al., 2020c; Khan et al., 2020d; Khan et al., 2021a; Khan et al., 2021b; Khan et al., 2021c). So far, a total of 173 SNPs comprising 119 missense mutations have been described in the human *PIR* gene and deposited to the database gnomAD (Karczewski et al., 2020).

The *PIR* gene is very polymorphic and is involved in tumorigenesis; however, at this stage, we are uncertain about the effects of the reported nsSNPs on protein structure and biological activities. Therefore, in the present study, with the help of various computational approaches, highly deleterious nsSNPs in the *PIR* gene will be identified, which profoundly affect the structure and function of *PIR* protein. This study is the first extensive *in silico* analysis of the *PIR* gene that can narrow down the candidate mutations for further validation and targeting for therapeutic purposes.

MATERIALS AND METHODS

Pirin Sequence and 3D Structure Data Collection

The online public resources were used to retrieve all the available data about the human *PIR* gene. All the experimentally reported single-nucleotide polymorphisms (SNPs) in the *PIR* gene were collected from an online database gnomAD (https://gnomad.broadinstitute.org/) (Karczewski et al., 2020), and the UniProt database (http://www.uniprot.org/) (Magrane, 2011) was used to retrieve the amino acid sequence (UniProt ID: O00625) that encodes for *PIR* protein. The already reported crystal structure (PDB ID: 6N0J) of *PIR* protein was obtained from the Protein Data Bank (http://www.rcsb.org/) (Rose et al., 2010).

DATA PROCESSING

Prediction of Functional Consequences of Non-Synonymous Single-Nucleotide Polymorphisms

Various online servers such as PredictSNP (Bendl et al., 2014), MAPP (Multivariate Analysis of Protein Polymorphism) (Chao et al., 2008), PhD-SNP (Predictor of human Deleterious Single Nucleotide Polymorphisms) (Capriotti and Fariselli, 2017), PolyPhen-2 (Polymorphism Phenotyping version 2) (Adzhubei et al., 2013), SIFT (Sorting Intolerant from Tolerant), SNAP (screening for non-acceptable polymorphisms) (Bromberg et al., 2008), and PANTHER (Protein ANalysis THrough Evolutionary Relationships) (Mi et al., 2019) were used to predict the functional effect of nsSNPs. The deleterious nsSNPs, as suggested by all servers, were selected for further analysis. PredictSNP (https://loschmidt.chemi.muni.cz/predictsnp1/) executes prediction with diverse tools and provides a more authentic and accurate substitute for the predictions provided by the individual integrated tool. The predictions by tools in the PredictSNP server are enhanced by experimental annotations from two databases (24). MAPP (http://mendel.stanford.edu/SidowLab/downloads/MAPP/) predicts the effect of all possible SNPs on the function of the protein by considering the physiochemical deviation present in a column of aligned protein sequence (Stone and Sidow, 2005). PhD-SNP (http://snps.biofold.org/phd- snp/phd-snp.html) predicts and divides nsSNPs into disease-related and neutral polymorphisms according to the score ranging from 0 to 1. This server considers SNPs as a disease associated with a score more than 0.5 by using a related program algorithm. The outputs of PhD-SNP depend on frequencies of wild and mutant residues, the conservation index of SNP position, and a number of sequences aligned (Capriotti et al., 2006). PolyPhen-2 (http://genetics.bwh.harvard.edu/pp2) predicts the effect of amino acid variation on protein structure and function. The PolyPhen output is represented with a score that ranges from 0 to 1. This online tool considers non-synonymous SNPs as deleterious, having a higher mutation score, while zero scores indicate no effect of amino acid substitution on protein function (Adzhubei et al., 2010). SIFT (http://sift.bii.a-star.edu.sg) is a program that predicts the effect of amino acid substitution on protein functions. The principles of SIFT predictions depend on the physicochemical properties of protein sequence and its homologies. SIFT classifies its output as deleterious or neutral according to the score ranging from 0 to 1 (0–0.05 as deleterious and 0.05–1 as neutral). (Sim et al., 2012). SNAP (https://rostlab.org/services/snap) is a neural network–based prediction server

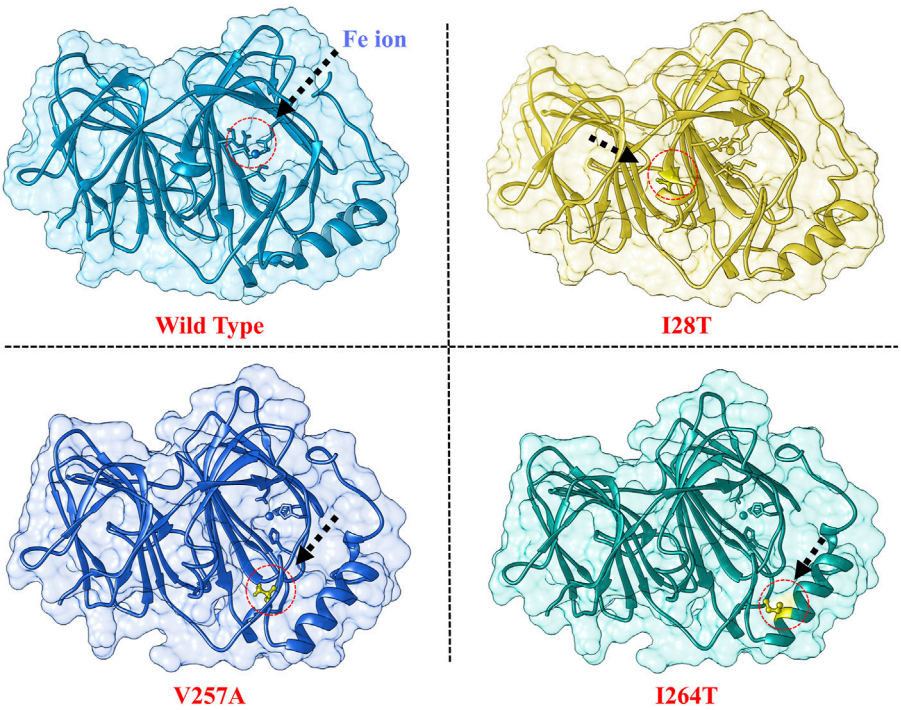

FIGURE 1 | Modeled 3D structures of the WT and I28T, V257A, and I264S mutants. The modeled mutations are encircled to show their position. The Fe ion binding is also shown.

that identifies the functional effects of amino acid sequence variants. The prediction score ranges from -100 (strongly neutral prediction) to 100 (strong effect prediction), which reflects the likelihood of the single amino acid mutation that may alter the native protein function (Hecht et al., 2015). PANTHER-PSEP (http://www.pantherdb.org/tools/csnpScoreForm.jsp) is an advanced online tool that predicts the non-synonymous mutations that have an important role in human diseases. PANTHER-PSEP uses a correlated but distinctive metric-based evolutionary conservancy. Homologous proteins are used to reconstruct the likely sequences of ancestral proteins at nodes in a phylogenetic tree, and the history of each amino acid can be traced back in time from its current state to estimate how long that state has been preserved in its ancestors.

Effect of Mutation on Structure Stability and Estimation of Evolutionary Conservation of Non-Synonymous Single-Nucleotide Polymorphisms

To analyze the effect of a mutation on protein stability, we used DynaMut (Rodrigues et al., 2020), a protein stability consensus predictor based on ENCoM's predicted vibrational entropy changes and the stabilization changes predicted by an mCSM's graph-based signature method. The degree of the evolutionary conservancy of protein sequence location correlates with the evolutionary degree, which is not the same for all amino acids in the corresponding protein. Positions of amino acids that change slowly are usually known to be conserved sites that are important for the structure and function of a protein.

Modeling of Wild Type and Variants of Pirin

The crystal structure of the *PIR* protein was extracted from the PDB (Entry ID: 6N0J). The protein structure was minimized using Chimera software [(Webb and Sali, 2016),33]. Moreover, the wild type (WT) structure was mutated by each one of the three most deleterious mutants predicted in the previous sections. The three structures of mutant (MT) proteins, such as I28T, V257A, and I264S, were modeled by making a point mutation in the wild-type (WT) protein structure using Chimera software. The WT and three MT structures are shown in **Figure 1**.

Molecular Dynamics Simulation

The AMBER18 (Mermelstein et al., 2018) package was used for molecular dynamics simulation to investigate the stability of WT and mutants of pirin (PIR) using the ff14SB force field (Maier et al., 2015). Molecular dynamics (MD) simulation was performed for a total of four systems, including one wild type (WT) and three mutants I28T, V257A, and I264S. For the solvation of each system in a rectangular box water model, TIP3P was used. With the help of counterions, addition neutralization was achieved. A two-step energy minimization procedure: the steepest decent minimizations of 6,000 cycles and conjugate gradient minimization of 3,000 cycles, was applied for minimization of each neutralized system. After minimization, these complexes were heated up to 300 K for 0.2 ns, and then we equilibrated the system with weak restraint and without restraint for 2 ns at 300 K, respectively. The temperature was controlled with a Langevin thermostat (Adzhubei et al., 2010) (26), and the procedure was run for 100 ns. Each simulation was repeated three

TABLE 1 | Processing of 119 missense variants by different servers predicted 24 mutations to be deleterious collectively. The predicted score by each server is also shown.

Variant	Predict SNP	MAPP	PhD-SNP	PolyPhen-1	PolyPhen-2	SIFT	SNAP	PANTHER	Outcome
E18G	0.869	0.508	0.676	0.744	0.811	0.792	0.622	0.662	DELETERIOUS
G19A	0.869	0.766	0.773	0.744	0.811	0.792	0.805	0.760	DELETERIOUS
I28T	0.869	0.461	0.676	0.594	0.550	0.792	0.720	0.780	DELETERIOUS
P38L	0.869	0.766	0.858	0.744	0.811	0.792	0.720	0.842	DELETERIOUS
H56Q	0.869	0.765	0.732	0.744	0.811	0.792	0.848	0.842	DELETERIOUS
H58R	0.869	0.919	0.875	0.744	0.811	0.792	0.885	0.874	DELETERIOUS
R59P	0.869	0.841	0.817	0.744	0.811	0.792	0.720	0.714	DELETERIOUS
G60V	0.869	0.913	0.875	0.744	0.811	0.792	0.848	0.874	DELETERIOUS
D77E	0.869	0.819	0.607	0.744	0.811	0.792	0.885	0.744	DELETERIOUS
H81P	0.869	0.656	0.588	0.744	0.453	0.792	0.720	0.718	DELETERIOUS
A95V	0.869	0.760	0.858	0.744	0.811	0.792	0.720	0.780	DELETERIOUS
G98S	0.869	0.573	0.875	0.744	0.811	0.792	0.848	0.744	DELETERIOUS
G98D	0.869	0.877	0.875	0.744	0.811	0.792	0.869	0.874	DELETERIOUS
H101Y	0.869	0.841	0.817	0.744	0.811	0.792	0.885	0.7145	DELETERIOUS
Q115K	0.869	0.856	0.773	0.744	0.811	0.792	0.848	0.744	DELETERIOUS
L116P	0.869	0.774	0.858	0.744	0.811	0.792	0.720	0.744	DELETERIOUS
G179V	0.869	0.751	0.817	0.744	0.562	0.792	0.555	0.744	DELETERIOUS
L220P	0.869	0.765	0.858	0.744	0.675	0.792	0.720	0.760	DELETERIOUS
E248A	0.869	0.718	0.875	0.744	0.647	0.792	0.720	0.686	DELETERIOUS
E248D	0.869	0.656	0.773	0.594	0.562	0.527	0.622	0.734	DELETERIOUS
G254V	0.869	0.842	0.773	0.744	0.811	0.792	0.848	0.780	DELETERIOUS
V257A	0.869	0.819	0.858	0.594	0.675	0.792	0.885	0.766	DELETERIOUS
M258I	0.869	0.559	0.607	0.594	0.600	0.527	0.805	0.698	DELETERIOUS
I264S	0.869	0.806	0.858	0.744	0.634	0.792	0.555	0.842	DELETERIOUS

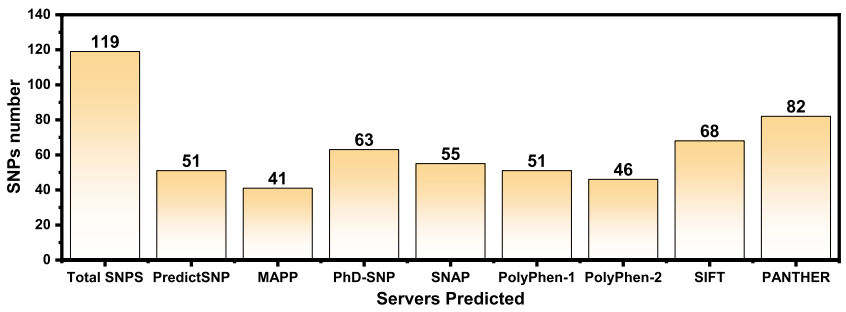

FIGURE 2 | The total number of predicted deleterious SNPs by each server is shown as bars. Each bar represents a specific server, and its predicted deleterious mutations are given on the top.

times. Long-range electrostatic interactions (Bromberg et al., 2008; Sim et al., 2012; Adzhubei et al., 2013; Hecht et al., 2015) were detected with the particle mesh Ewald method (Petersen, 1995) using a cut-off distance of 10.0 Å. The SHAKE method was applied for covalent bond treatment (Mi et al., 2019). The MD simulation production step was performed on the GPU-supported PMEMD code for each system (Glaser et al., 2003; Stone and Sidow, 2005), and the trajectories were analyzed on the CPPTRAJ package in Amber18.

Principal Component Analysis and Gibbs Free Energy Calculation

Principal component analysis (PCA) was utilized for the calculation of high-amplitude fluctuations within the protein (Berezin et al., 2004; Capriotti et al., 2006). The CPPTRAJ package calculated the covariance matrix based on Cα coordinates. Eigenvectors and eigenvalues were calculated by diagonalizing the covariance matrix. 5,000 snapshots from the trajectory of each system were used to get PCA calculations. Eigenvectors and eigenvalues indicate the direction of motion and mean square fluctuation, respectively. PC1 and PC2 were used for plotting to monitor the motion. The lowest energy stable state was determined by the free energy landscape (FEL) and is indicated by deep valleys on plot, whereas the intermediate state is shown by boundaries between deep valleys (Xu et al., 2017; Adzhubei et al., 2010). In this study, FEL calculations based on PC1 and PC2 were obtained by

$$\Delta G\ (PC1,\ PC2)\ =\ -KBTln\ P\ (PC1,\ PC2 \qquad (1)$$

where the reaction coordinates are taken by PC1 and PC2, K_B denotes the Boltzmann constant, and P (PC1, PC2) shows the

TABLE 2 | A list of 24 highly deleterious mutations was processed to identify the highly destabilizing mutations.

Index	Mutation	ΔΔG mCSM	Outcome
1	E18G	−1.063	Destabilizing
2	G19A	−0.265	Destabilizing
3	**I28T**	**−2.374**	**Highly destabilizing**
4	P38L	−0.839	Destabilizing
5	H56Q	−0.777	Destabilizing
6	R59P	−1.518	Destabilizing
7	H58R	−1.986	Destabilizing
8	G60V	−0.63	Destabilizing
9	D77E	−0.822	Destabilizing
10	H81P	0.715	Stabilizing
11	A95V	−0.937	Destabilizing
12	G98D	−1.812	Destabilizing
13	G98S	−1.53	Destabilizing
14	H101Y	−0.191	Destabilizing
15	Q115K	−0.244	Destabilizing
16	L116P	−1.237	Destabilizing
17	G179V	−0.66	Destabilizing
18	L220P	−1.406	Destabilizing
19	E248A	−0.764	Destabilizing
20	E248D	−0.693	Destabilizing
21	G254V	−0.205	Destabilizing
22	**V257A**	**−2.157**	**Highly destabilizing**
23	M258I	−0.996	Destabilizing
24	**I264S**	**−2.856**	**Highly destabilizing**

Based on ΔΔG, the mCSM server predicted I28T, V257A, and I264S as highly destabilizing, while the rest were classified as destabilizing only. Bold are highly destabilizing mutations which were subjected to MD simulation.

probability distribution of the system along with the first two principal components.

RESULTS AND DISCUSSION

Identification of Deleterious Non-Synonymous Single-Nucleotide Polymorphisms

The online public resources were used to retrieve all the available data of the human PIR gene. According to the information obtained from the online database gnomAD, there were a total of 173 SNPs in the *PIR* protein. Of those, 119 SNPs were identified as non-synonymous. These 119 SNPs were submitted to different online servers for identification of the deleterious mutations. First, the SNPs were submitted to PredictSNP and MAPP servers, and only 51 and 41 SNPs were found as deleterious, respectively. The nsSNPs were then submitted to PhD-SNP and SNAP online tools, and 63 and 55 SNPs were found as deleterious, respectively. The other online servers such as PolyPhen-1, PolyPhen-2, SIFT, and PANTHER analyzed the nsSNPs and predicted that, out of 119 SNPs, only 51, 46, 68, and 80 were deleterious, respectively. All the nsSNPs were selected for further analysis that were predicted as deleterious consistently by all the above online servers as shown in **Table 1**. The total number of predicted deleterious SNPs by each server is given in **Figure 2**.

Effect of Mutation on Pirin Protein Structure Stability

To calculate the stability changes upon mutations, an online server mCSM was used, which reported the average changes ranging from 0.715 to −2.856 kcal/mol. Mutations, such as V257A with a stability fold change of -2.157 kcal/mol, I28T with a stability fold change of -2.374 kcal/mol, and I264S with a stability fold changes of -2.856 kcal/mol, were found to be highly destabilizing for the *PIR* protein structure. However, the mutation H81P with a stability fold change of 0.715 kcal/mol has the opposite effect (i.e., stability) and does not induce major changes in the protein structure (**Table 2**). The RMSDs between the WT and the three mutants are shown as a superimposed structure in **Figure 3**. The highly destabilizing mutations identified by mCSM were analyzed by DynaMut to check the effect of these mutations on the structure flexibility. As shown in **Figure 4**, the mutations I28T, V257A, and I264S produced higher flexibility in the protein structure. These results are clearly pointing out the importance of these three mutations. These changes in flexibility (red) and rigidity (blue) are mapped onto the corresponding protein structure and presented in **Figure 4**.

Dynamics Stability and Residual Flexibility of the Wild and Mutant Structures

To estimate the impact of the specific mutant (I28T, V257A, and I264S) and WT, we calculated the RMSD (root mean square deviation) from the MD trajectory. We used 5,000 structures

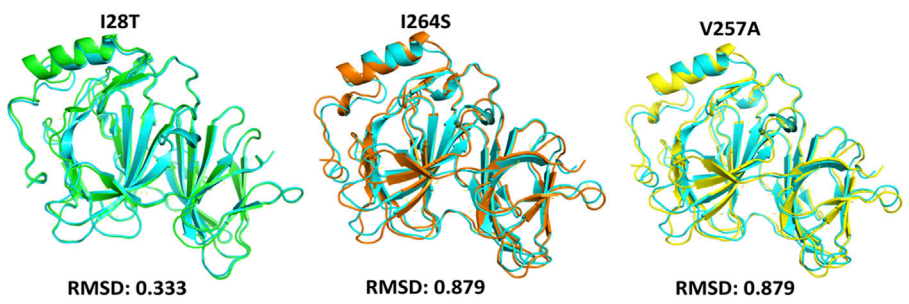

FIGURE 3 | Superimposed structure of WT *PIR* protein (cyan) with mutants I28T (green), I264S (orange), and V257A (yellow). The RMSD of each superimposition was reported to be 0.333 Å (I28T) and 0.879 Å (I264S, V257A).

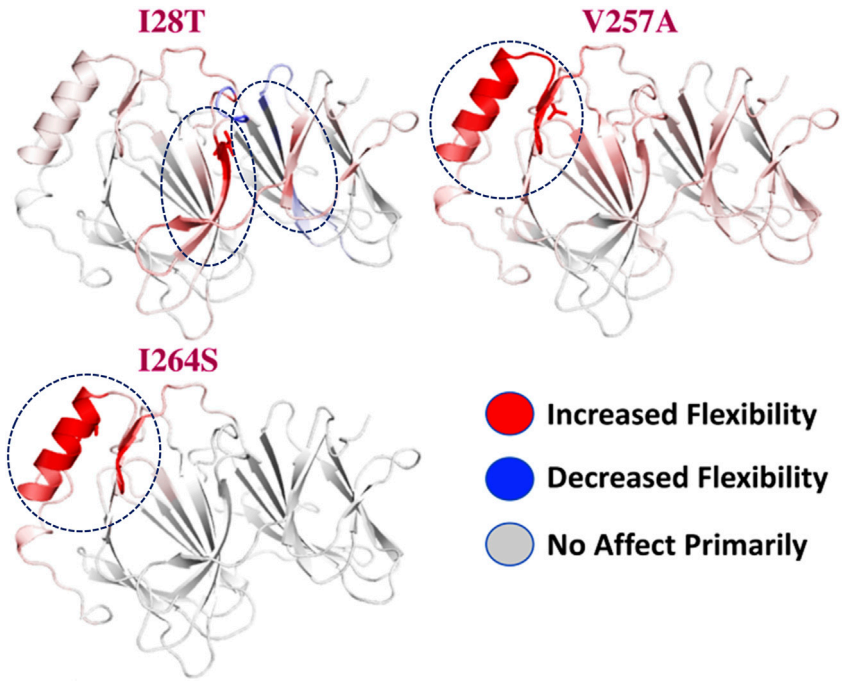

FIGURE 4 | Effect of mutations on structural dynamics flexibility. Changes in flexibility (red) and rigidity (blue) mapped onto the corresponding protein structure are shown.

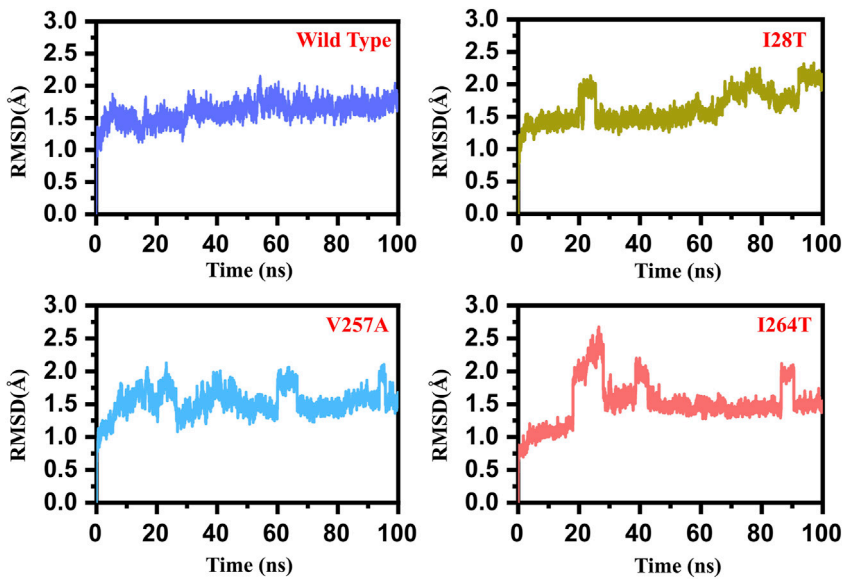

FIGURE 5 | RMSD graph of the WT and mutants (I28T, V257A, and I264S). The *x*-axis shows the time in nanoseconds, while the *y*-axis shows the RMSD in angstrom. **(A)** Wild type; **(B)** I28T; **(C)** V257A; **(D)** I264S.

from the MD trajectory as a function of time. In the case of the WT, the RMSD remained stable during the 100 ns simulation time. No significant convergence was observed. The system reached the stability at 1.3 Å. The average RMSD was reported to be 1.25 Å. Overall, the system seems to be stable with no significant convergence during the 100 ns simulation. On the contrary, the I28T mutation showed significant convergence at different intervals. Initially, the structure continued to proceed stably until 20 ns, but after the system faced convergence, the RMSD increased from 1.5 to 2.0 Å.

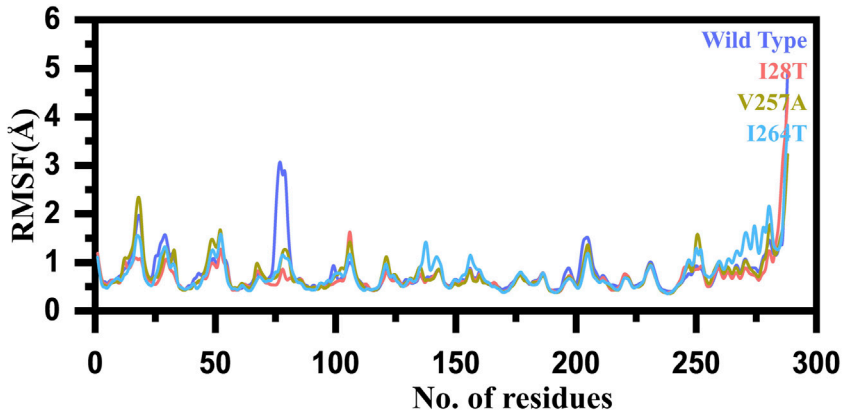

FIGURE 6 | RMSF graph of the WT and mutants (I28T, V257A, and I264S). The *x*-axis shows the number of residues, while the *y*-axis shows the RMSF in angstrom.

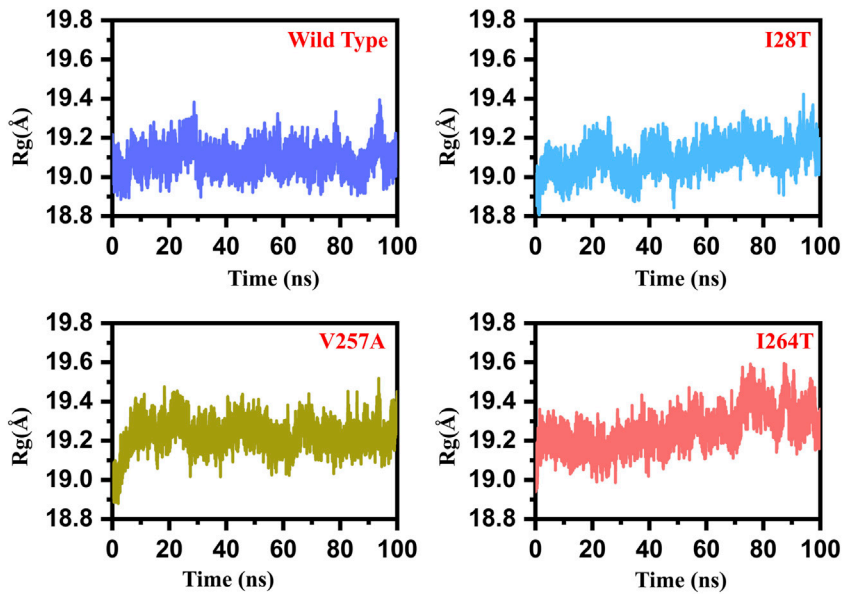

FIGURE 7 | Rg graph of the WT and mutants (I28T, V257A, and I264S). The *x*-axis shows the time in nanoseconds, while the *y*-axis shows the RMSD in angstrom.

Afterward, the RMSD decreased and remained uniform until 70 ns, but the structure faced significant perturbation and the RMSD increased again until 100 ns. The major convergence was observed specifically between 75 and 90 ns. The average RMSD (1.8 Å) also remained higher than that in the wild type. This shows that the I28T mutation has caused a significant structural stability shift and needs longer simulation to gain the equilibrium. Furthermore, the V257A mutation also induced significant structural stability changes. The RMSD remained higher during the 100 ns simulation. Initially, the RMSD increased until 1.25 Å and then continued to increase until 20 ns. Afterward, an abrupt decrease was observed at 22 ns, and then again, the RMSD increased. The RMSD between 60 and 80 ns significantly converged, and the average RMSD between 60 and 80 ns was observed to be 2.0 Å. The RMSD then decreased and remained uniform until 95 ns, but then again, the structure converged and the RMSD increased. Hence, the V257A mutation has caused significant structural perturbation, and the stability significantly shifted as compared to that of the wild type. I264S was reported to be the most destabilizing mutation among the list of 24 non-synonymous mutations reported to be deleterious. The results here are uniform with the mCSM server. The mutation has induced significant stability transition and perturbation. Initially until 20 ns, the RMSD remained uniform, but a sudden convergence increased the RMSD up to 2.5 Å. Later on, the RMSD decreased for a short period of time, and then significant convergence was observed between 35 and 40 ns. The RMSD then remained lower and uniform until 85 ns. The structure then faced significant perturbation, and the RMSD increased again up to 2.0 Å. The average RMSD for

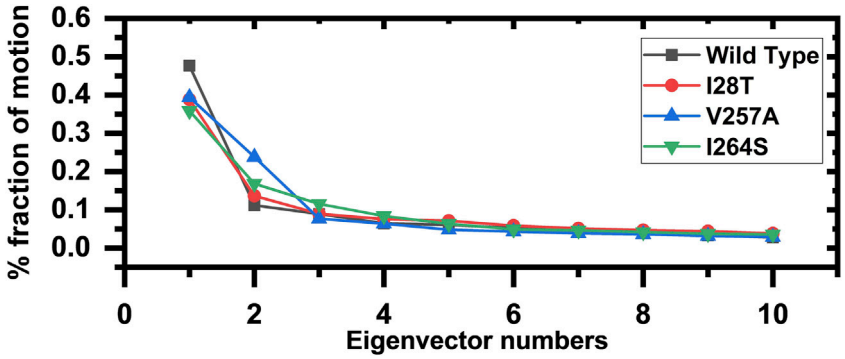

FIGURE 8 | Fractions of the first ten eigenvectors. Using the MD trajectory, the fraction of motions is calculated and given in percentage against the eigenvector numbers.

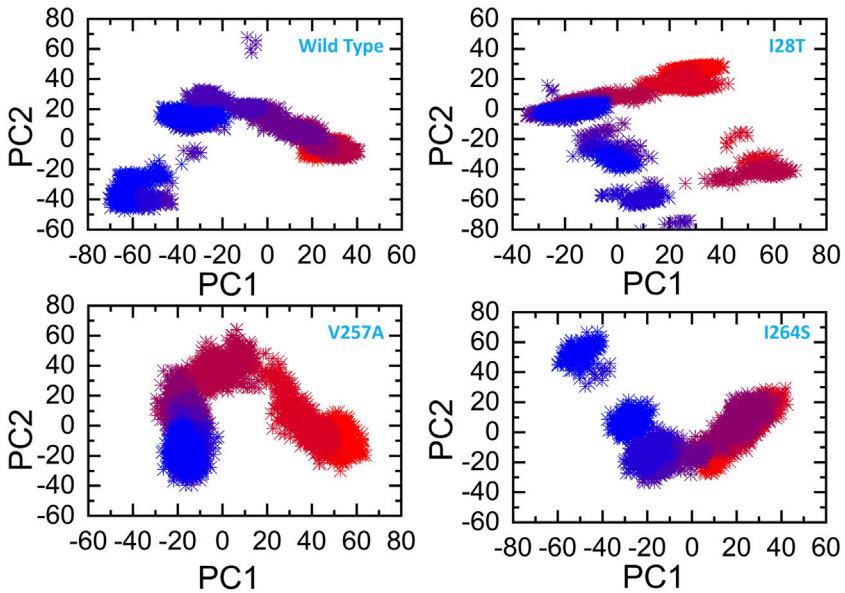

FIGURE 9 | Principal component analysis of all the systems, including the WT and the three mutants. The first two principal components (PC1 and PC2) are used for the projection of motion in the space phase at 300 K.

I264S was reported to be 2.2 Å. Thus, these results signify that the mutations have caused significant structural destability and internal dynamics of the protein. The RMSD graph of the WT and mutants (I28T, V257A, and I264S) is shown in **Figure 5**. The *x*-axis shows the time in nanoseconds, while the *y*-axis shows the RMSD in angstrom.

Furthermore, to estimate the impact of the mutation on the residual flexibility, we calculated the RMSF (root mean square fluctuation) as a function of residues. Overall, the residual flexibility showed more similar fluctuation except in few regions. In the case of V257A, the region between 15 and 25 showed higher fluctuation than the others. In addition, the region between 72 and 85 in the WT possesses higher fluctuation than the mutants. Thus, this shows that this region is significantly affected by the mutation induction. In the case of I264S, specifically the region between 140 and 150 showed higher fluctuation. Furthermore, this mutation also increased the fluctuation of the region between 250 and 280, thus causing significant internal dynamics fluctuation. These results show that the mutation has affected different regions of the protein to increase or decrease the flexibility. The RMSF graph of the WT and mutants (I28T, V257A, and I264S) is shown in **Figure 6**. The *x*-axis shows the number of residues, while the *y*-axis shows the RMSF in angstrom.

Structural Compactness Estimation of the Wild and Mutant Structures

In order to calculate the compactness of all the WT and mutant (I28T, V257A, and I264S) systems, R_g (radius of gyration) was calculated. The stability of the complexes formed also depended on the compactness of the structure. From **Figure 7**, it can be

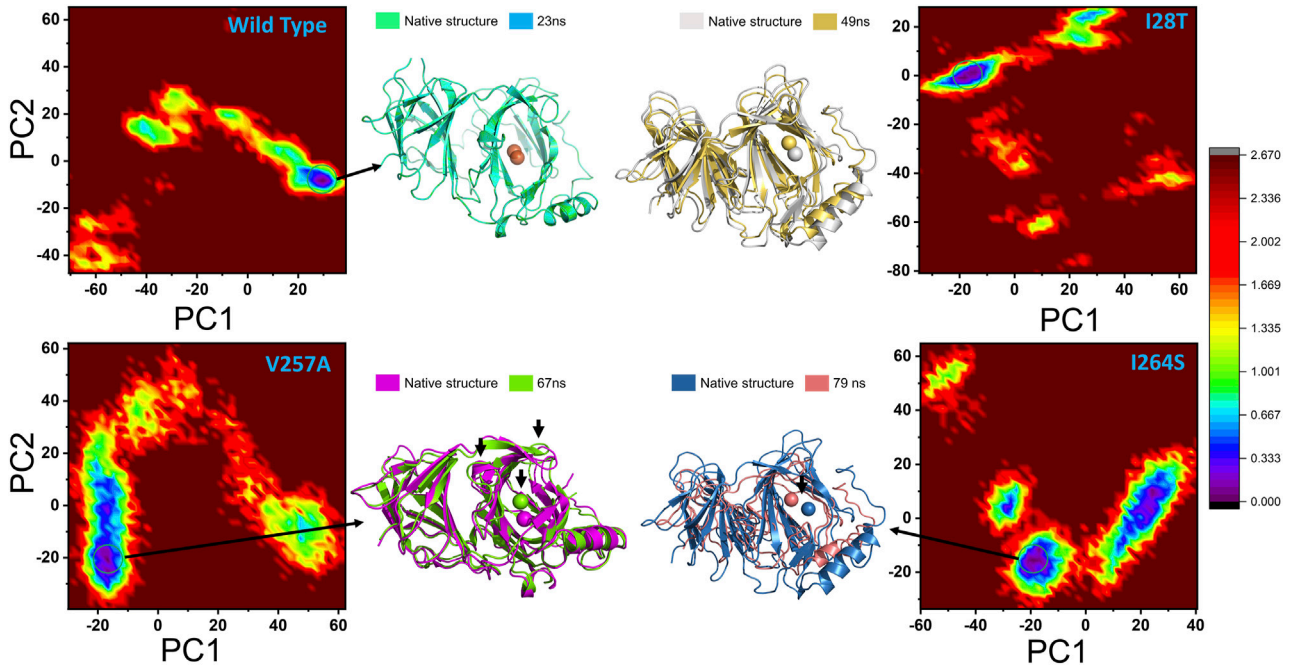

FIGURE 10 | Conformational changes during the molecular dynamics simulation are represented through the FEL. PC1 and PC2 are used to map the energy minima and extract the variations due to a specific mutation.

easily observed that the average R_g value for all the systems is between 19.0 and 19.4 Å. In the case of wild type, the R_g value remained uniform until 100 ns. The average value for the WT was observed to be 19.0 Å. In the case of I28T, the system remained relatively less compact than the wild type. The average R_g value was reported to be 19.0 Å for the first 52 ns, and then the R_g value continued to increase and reached 19.2 Å during the simulation time. In the case of V257A, the R_g value remained lower until 5 ns. The R_g then continued to increase until 100 ns. The R_g value for the rest of 95 ns remained 19.3 Å. The R_g for I264S started from 19.2 Å and continued to increase. After reaching 30 ns, the R_g value increased to 19.3 Å and increased further. After 70 ns, the R_g value further increased to 19.5 Å and continued until 100 ns. These results significantly justify that the mutation has different compactness than the WT during the simulation. The R_g graph of the WT and mutants (I28T, V257A, and I264S) is shown **Figure 7**. The *x*-axis shows the time in nanoseconds, while the *y*-axis shows the R_g in angstrom.

Dimensionality Reduction and Clustering the Protein Motions

To describe the protein motion and clustering of the related structural frames, principal component analysis (PCA) was performed. PCA is a statistical approach that incorporates a smaller number of uncorrelated variables called principal components into several correlated variables. The eigenvectors were measured and are provided in **Figure 8** to comprehensively explain the effect of the substitution on the protein motion. From the PCs, we can understand the overall and internal motions. In the wild type, the total contributed variance by the first three eigenvectors to the total motions was reported to be 47%, while in the case of I28T, the variance by the three eigenvectors was observed to be 38%, and for V257A, it was observed to be 39%. In the other mutation such as I264S, the variance by the first three eigenvectors was reported to be 35%.

The other eigenvectors have shown localized or overall motions. Hence, it is confirmed that the mutations have impacted the total trajectory motion and, thus, internal dynamics behavior. To further gain convincible attributes, the first two PCs, i.e., PC1 and PC2, were drawn against each other. Different colors (red to blue) reflect the conformational transition from one to another. Each dot in **Figure 9** depicts a single frame of the trajectory. As compared to the WT, the mutant complexes covered a lower region of the phase space except in V257A and I264S. Together, these observations suggest that mutations had a substantial influence on the structure that has contributed to pirin (PIR) destabilization.

Conformational changes induced by a particular mutation during the MD simulation were explored through the FEL. PC1 and PC2 were used to map the energy minima and extract the variations due to a specific mutation. In the case of the wild type, the lowest energy minima were reached at 23 ns. **Figure 10** shows that, during the simulation, no structural perturbation was experienced in the wild type. On the contrary, in the three mutations, destabilization of the Fe ion was observed. The energy minima separated by subspace in each mutant complex were reached at 49 ns (I28T), 67 ns (V257A), and 79 ns (I264S). In addition, the cavity surrounding the Fe ion also exhibits a dynamic structure in all the mutants. The beta sheet covering the

Fe ion from the top and the loop on the alternate side changed their orientations, and an opening–closing switch-like pattern was observed. In addition, the flipping of beta sheets in the mutant complexes was most frequently observed in the mutant complexes. All the FEL graphs of the wild type, I28T, V257A, and I264S are given in **Figure 10**.

CONCLUSION

PIR is an oxidative stress sensor from the functionally diverse superfamily of cupin. This protein is suggested to have biological relevance in cancer development and thus remains a novel research area. Being polymorphic, its oncogenic activity is a hot topic of discussion in the recent past. The work reported herein attempted to use an extensive computational framework to screen all potential mutations of the protein and identify deleterious mutants that could affect protein structure stability and ultimately functionality. The work predicted 119 missense variants by different servers and reported 24 deleterious mutations consistently reported by all available mutation predictor servers. Furthermore, it was highlighted that the three mutations I28T, V257A, and I264S are most destabilizing and confer structure flexibility to the PIR protein. To sum up, the study provides structural basis for each mutation-induced conformational change and disclosed a possible way for the mutations' role in the progression of Breast Cancer (BC); thus, PIR acts a potential therapeutic target or a biomarker in the future ahead.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ Supplementary Material.

AUTHOR CONTRIBUTIONS

MS, MT, and SS performed data retrieval. MS, MT, SS, SA, SSA, AA, FA, MW, and KA were responsible for data analysis. HK, FA, and WK were responsible for graphics and writing. AA, FA, MW, and KA have contributed to drafting and finalizing the manuscript.

FUNDING

The article-processing charges were funded by Qassim University, Saudi Arabia. We acknowledge their help.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a past co-authorship with several of the authors (SS, SSA, and MS).

frontiers in Molecular Biosciences

ORIGINAL RESEARCH
published: 21 September 2021
doi: 10.3389/fmolb.2021.752154

Prognostic Effect of Microenvironment Phenotype in Triple-Negative Breast Cancer: Biomarker Analysis of a Prospective Trial

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Background: The microenvironment of triple-negative breast cancer (TNBC) can be divided into three clusters based on bioinformatics-based immunogenomic analysis: the "immune-desert" cluster, the "innate immune-inactivated" cluster, and the "immune-inflamed" cluster. The immune-inflamed cluster is considered as "hot tumor" while the other two are considered as "cold tumor".

Methods: To investigate the prognostic effect of microenvironment phenotypes on TNBC, we compared relapse-free survival (RFS) of different phenotypes in 100 patients with RNA sequencing-based expression data from the PATTERN trial (NCT01216111, published in JAMA Oncol 2020), which indicated a superior efficacy of adjuvant paclitaxel-plus-carboplatin regimen compared to the regimen of cyclophosphamide/epirubicin/fluorouracil followed by docetaxel for TNBC. We also analyzed the efficacy of the two regimens for different immune phenotypes to explore potential treatment strategies.

Results: No significant difference in RFS was observed between the "hot tumor" and the "cold tumor" (hazard ratio [HR] = 0.68, 95% confidence interval [CI] 0.28–1.66, P = 0.40). However, the "hot tumor" subtype was associated with significantly longer RFS in node-positive patients (HR = 0.27, 95%CI 0.07–0.97, P = 0.03). Consistently, a similar trend to improved RFS of the "hot tumor" phenotype was detected in patients with stage pT2-3 tumors (HR = 0.29, 95%CI 0.06–1.30, P = 0.08). Furthermore, no significant difference in RFS between the two treatment arms was observed in patients with "hot tumor" (HR = 0.39, 95% CI 0.08–2.01, P = 0.24) or "cold tumor" (HR = 1.05, 95% CI 0.39–2.82, P = 0.92).

Conclusion: The microenvironment phenotype in TNBC might have prognostic significance to patients with a high risk of recurrence. The association of the microenvironment phenotypes with the efficacy of adjuvant chemotherapy for TNBC remains to be further studied.

Keywords: triple-negative breast cancer, microenvironment phenotype, biomarker, trial, immune

OPEN ACCESS

Edited by:
Dongqing Wei,
Shanghai Jiao Tong University, China

Reviewed by:
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Specialty section:
This article was submitted to Molecular Diagnostics and Therapeutics, a section of the journal Frontiers in Molecular Biosciences

Received: *02 August 2021*
Accepted: *31 August 2021*
Published: *21 September 2021*

Citation:
Zhu S-Y, Ma D, Shao Z-M and Yu K-D (2021) Prognostic Effect of Microenvironment Phenotype in Triple-Negative Breast Cancer: Biomarker Analysis of a Prospective Trial. Front. Mol. Biosci. 8:752154. doi: 10.3389/fmolb.2021.752154

INTRODUCTION

Triple-negative breast cancer (TNBC) accounts for 15–20% of breast cancers that lack estrogen receptor (ER) and progesterone receptor (PR) expression and human epidermal growth factor 2 (HER2) amplification (Perou et al., 2000; Harbeck and Gnant, 2017). Higher risk of relapse and metastasis and lack of therapeutic targets are major problems in TNBC treatment at present (Bianchini et al., 2016; Denkert et al., 2017). Compared with other subtypes of breast cancer, TNBC usually has higher immunogenicity (Lehmann et al., 2011; Burstein et al., 2015). Immune infiltration in the tumor microenvironment (TME) is associated with response to treatment and prognosis of TNBC (Loi et al., 2014; Denkert et al., 2018). Therefore, efforts have been made to explore immunotherapeutic strategies for patients with TNBC. Recent research has shown that the application of immune checkpoint blockade (ICB) may benefit metastatic TNBC (Schmid et al., 2018).

To systemically characterize the impact of the TNBC microenvironment on prognosis and immunotherapy, we have classified the TNBC microenvironment phenotypes into three heterogeneous clusters taking advantage of the expression data of 386 TNBC patients from Fudan University Shanghai Cancer Center (FUSCC): the "immune-desert" cluster with low microenvironment cell infiltration; the "innate immune-inactivated" cluster with resting innate immune cells and nonimmune stromal cells infiltration; and the "immune-inflamed" cluster with abundant adaptive and innate immune cells infiltration (Xiao et al., 2019). The "immune-inflamed" cluster is considered as "hot tumor" while the other two clusters are considered as "cold tumor".

To further investigate the prognostic effect of the TNBC microenvironment phenotypes and their association with the efficacy of different adjuvant chemotherapy regimens, we conducted a biomarker analysis of the patients with immunogenomic data on microenvironment phenotypes from the PATTERN trial (NCT01216111). The randomized multicenter phase III PATTERN trial compared six cycles of paclitaxel plus carboplatin (PCb) with a standard-dose regimen of three cycles of cyclophosphamide, epirubicin, and fluorouracil followed by three cycles of docetaxel (CEF-T) in the adjuvant setting of operable TNBC, indicating a superior efficacy of the carboplatin-containing regimen compared to the anthracycline/taxane regimen (Yu et al., 2020). A total of 100 patients in the PATTERN cohort with expression data from RNA sequencing or HTA 2.0 microarray have been involved in clustering TNBC microenvironment phenotype mentioned above. Here, we analyzed the clinical characteristics and long-term survival data of these patients to explore clues for potential treatment strategies of adjuvant chemotherapy or immunotherapy for TNBC.

MATERIALS AND METHODS

Study Design

The design and conduct of the PATTERN trial were described elsewhere previously (Yu et al., 2020). In brief, between July 2011 and April 2016, 647 women with operable, primary invasive TNBC after definitive surgery at nine cancer centers and hospitals in China were randomly assigned to two treatment groups: 322 in the CEF-T group and 325 in the PCb group. The primary endpoint was disease-free survival (DFS). Secondary endpoints included overall survival distant DFS, relapse-free survival (RFS), DFS in patients with germline variants in BRCA1/2 or homologous recombination repair-related genes, and toxicity. The independent institutional review board of the participating centers approved the study protocol. We performed the study according to the International Conference on Harmonisation Good Clinical Practice guidelines and ethical principles of the Declaration of Helsinki. All patients provided written informed consent.

Patient Samples

As mentioned above, a total of 100 patients with RNA sequencing data or HTA 2.0 microarray data in the PATTERN cohort were enrolled in the previous immunogenomic analysis of TNBC microenvironment phenotypes clustering. There were 47 patients in the PCb arm and 53 patients in the CEF-T arm involved, respectively. Detailed inclusion criteria for the analysis were as follows: 1) female patients; 2) unilateral invasive ductal carcinoma; 3) pathologic examination of the ER, PR, and HER2 status performed by the Department of Pathology at FUSCC through immunochemical analysis and *in situ* hybridization (for HER2 status only); 4) patients with no evidence of metastasis at the time of diagnosis; and 5) sufficient frozen tissue for further research. More detailed information regarding the sample processing and sequencing data generation is described previously (Xiao et al., 2019). All data can be viewed in The National Omics Data Encyclopedia (http://www.biosino.org/node) by pasting the accession (OEP000155) into the text search box or through the URL: http://www.biosino.org/node/project/detail/OEP000155. The HTA 2.0 microarray data is also available in GSE76250 and the RNA sequencing data is available in SRP157974.

Microenvironment Phenotypes Clustering

The detail of microenvironment phenotypes clustering and relevant data processing was described elsewhere (Xiao et al., 2019). In brief, we firstly constructed a compendium of 364 genes to represent 24 microenvironment cell subsets by referring to two gene signatures, CIBERSORT (Newman et al., 2015) and MCP-Counter (Becht et al., 2016). Signatures for types 1, 2, and 17 T helper cells and myeloid-derived suppressor cells were also constructed according to a published article (Angelova et al., 2015). Then we used the "GSVA" function in R to calculate the single sample gene set enrichment analysis (ssGSEA) score to measure the abundance of each cell subset in the samples. Adjusted scores were calculated as the enrichment scores divided by the (1 - tumor purity), which was calculated by the allele-specific copy-number analysis of tumors (Van Loo et al., 2010). Subsequently, k-means clustering was performed to classify the TNBC microenvironment phenotypes into three clusters: the "immune-desert" cluster, the "innate immune-inactivated" cluster, and the "immune-inflamed" cluster. The

TABLE 1 | Clinicopathologic characteristics by microenvironment phenotypes.

Characteristics	Cold tumor (n = 63)		Hot tumor (n = 37)		*p* Value
	No	%	No	%	
Age at diagnosis					
Median (IQR),years	53 (46–61)		53 (49–56)		0.82
Chemotherapy regimen					
PCb	29	46.0	18	48.6	0.80
CEF-T	34	54.0	19	51.4	
Pathologic tumor size					
pT1	26	41.3	19	51.4	0.33
pT2-pT3	37	58.7	18	48.6	
Nodal status					
Negative	41	65.1	18	48.6	0.11
Positive	22	34.9	19	51.4	
Histological grade					
I-II	31	49.2	8	21.6	0.01
III	32	50.8	29	78.4	
Ki67 proliferation index (%)					
≤14	7	11.1	1	2.7	0.13
>14	56	88.9	36	97.3	
Adjuvant radiation					
Yes	16	25.4	15	40.5	0.11
No	47	74.6	22	59.5	

CEF-T, fluorouracil, epirubicin, and cyclophosphamide followed by docetaxel; IQR, interquartile range; PCb, paclitaxel and carboplatin.

"immune-desert" cluster and the "innate immune-inactivated" cluster were referred as "cold tumor" while the "immune-inflamed" cluster was referred as "hot tumor".

Statistical Analysis

The primary endpoint of this analysis was RFS. The RFS events were defined as the first recurrence of locally, regionally, or distantly invasive disease, a diagnosis of contralateral breast cancer, or death from any cause. The Kaplan-Meier method was used to estimate the distributions of survival outcomes, with the log-rank test evaluating differences of survival outcome. Cox proportional hazards model was used to obtain hazard ratios (HR) and 95% confidence intervals (CI). Differences of continuous and categorical factors were assessed by the Wilcoxon rank-sum test and the $\chi 2$ test (or Fisher exact test when necessary). All statistical tests were two-tailed, with the significant level being set at $p < 0.05$. Data were analyzed with STATA version 16.0 and R version 3.4.2.

RESULTS

Patient Samples and Clinical Data

Clinicopathologic characteristics of the 100 patients involved are demonstrated in **Table 1**. There were 47 patients in the PCb arm and 53 patients in the CEF-T arm, respectively. The median age of these patients was 53 years (interquartile range, 47–59 years) at the time of PATTERN study entry. Among them, 43 patients belonged to the "immune-desert" cluster. Twenty and 37 patients belonged to the "innate immune-inactivated" cluster and the "immune-inflamed" cluster, respectively. Therefore, 63 patients with "cold tumor" and 37 patients with "hot tumor" were included in the analysis. **Figure 1** depicted the details of the distribution of the microenvironment phenotype (**Figure 1A**), the FUSCC subtype (Jiang et al., 2019) (**Figure 1B**) and the intrinsic subtype (Parker et al., 2009) (**Figure 1C**) of these enrolled patients.

Baseline characteristics of the patients of the two types are similar except that patients with "hot tumor" had relatively higher tumor histological grade than the patients with "cold tumor" (P = 0.01). There was no significant difference in chemotherapy regimens for patients of different microenvironment phenotypes as well. Compared with baseline characteristics of the whole PATTERN cohort, more patients enrolled in this analysis were node-positive (**Table 2**) in that these patients with a relatively greater tumor burden were more likely to provide sufficient frozen tissue and fulfill the criteria for further pathologic examination. 5

Prognostic Significance of Microenvironment Phenotypes in TNBC

Considering the important role of TME in tumor progression, we investigated the prognostic significance of different TNBC microenvironment phenotypes taking advantage of the long-term survival data of the 100 patients from the PATTERN cohort. Firstly, we examined the association of microenvironment phenotypes with RFS status. The distribution of different types of the microenvironment in TNBC was similar between patients with different RFS statuses (P = 0.46). No significant difference in RFS was either detected between the patients with "hot tumor" and the patients with "cold tumor" (**Figure 2A**, HR = 0.68, 95% CI 0.28–1.66, P = 0.40).

However, we found that the TNBC microenvironment phenotypes were significantly associated with RFS status in the node-positive patients (P = 0.04). In contrast, no significant

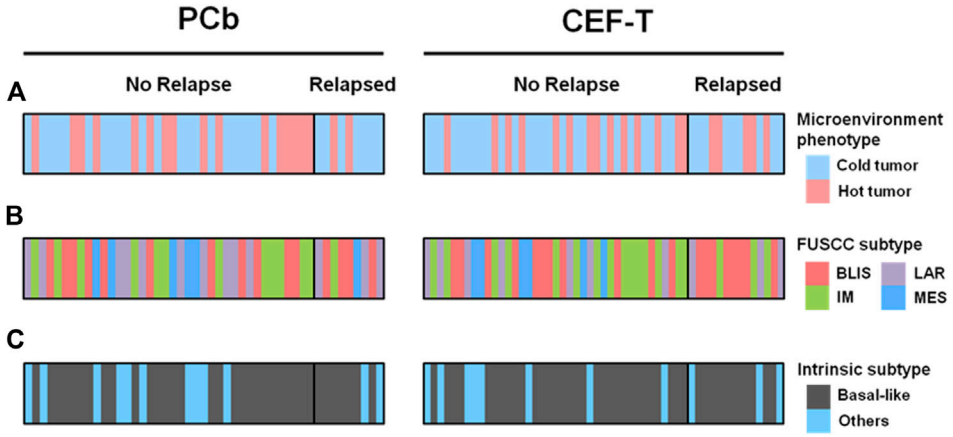

FIGURE 1 | Microenvironment phenotype and other different subtypes by treatment cohorts. **(A)** Microenvironment phenotype, **(B)** FUSCC subtype, and **(C)** intrinsic subtype of the patients enrolled in the analysis. BLIS indicates basal-like and immune-suppressed; CEF-T, fluorouracil, epirubicin, and cyclophosphamide followed by docetaxel; FUSCC, Fudan University Shanghai Cancer Center; IM, immunomodulatory; LAR, luminal androgen receptor; MES, mesenchymal-like; PCb, paclitaxel and carboplatin.

TABLE 2 | Characteristics of the PATTERN cohort and the patients undergoing microenvironment phenotypes clustering.

Characteristics	PATTERN cohort (n = 647)		Microenvironment phenotypes (n = 100)		*p* Value
	No	%	No	%	
Age at diagnosis					
Median (IQR),years	51 (44–57)		53 (47–59)		0.13
Chemotherapy regimen					
PCb	325	50.0	47	47.0	0.55
CEF-T	322	50.0	53	53.0	
Pathologic tumor size					
pT1	351	54.2	45	45.0	0.08
pT2-pT3	296	45.8	55	55.0	
Nodal status					
Negative	481	74.3	59	59.0	<0.01
Positive	166	25.7	41	41.0	
Histological grade					
I-II	177	27.4	39	39.0	0.02
III	470	72.6	61	61.0	
Ki67 proliferation index (%)					
≤14	80	12.4	9	8.0	0.32
>14	567	87.6	92	92.0	
Adjuvant radiation					
Yes	296	45.7	31	31.0	0.01
No	351	54.3	69	69.0	

CEF-T, fluorouracil, epirubicin, and cyclophosphamide followed by docetaxel; IQR, interquartile range; PCb, paclitaxel and carboplatin. Among the patients receiving PCb in this analysis, 20 (42.6%) patients were node-positive and 29 (61.7%) patients were in stage pT2-3. Among the patients receiving CEF-T in this analysis, 21 (39.6%) patients were node-positive and 26 (49.1%) patients were in stage pT2-3. The PCb group had more advanced disease compared with the CEF-T group.

association was found between microenvironment phenotypes and RFS status in the node-negative patients (P = 0.47). Consistently, in the node-positive patients, a significantly better RFS was observed in the patients with "hot tumor" than the patients with "cold tumor" (**Figure 2B**, HR = 0.27, 95% CI 0.07–0.97, P = 0.03). There was no evidence of different RFS outcomes between the two phenotypes in the patients without lymph node metastasis (HR = 1.57, 95% CI 0.44–5.61, P = 0.48).

Subsequently, we investigated the prognostic relevance of microenvironment phenotypes in patients with different pathological tumor sizes. Given the limited number of cases enrolled in the analysis, a borderline significant association was indicated between the microenvironment phenotype with RFS status in the patients with tumor in stage pT2-3 (P = 0.09). Nevertheless, either of the microenvironment phenotypes was significantly related to RFS status in the patients with tumor in

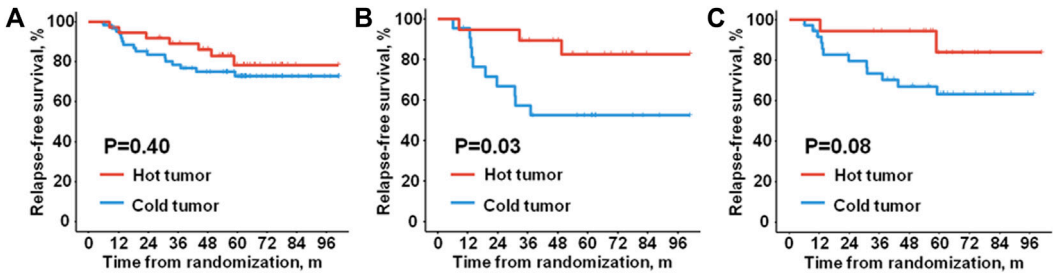

FIGURE 2 | Relapse-free survival of different microenvironment phenotypes. Kaplan-Meier plots show relapse-free survival of **(A)** all the enrolled patients, **(B)** the node-positive patients, and **(C)** the patients with tumors in stage pT2-3.

TABLE 3 | Multivariate analysis of microenvironment phenotypes in TNBC.

	Node-positive			Node-negative			Tumor stage pT2-3			Tumor stage pT1		
Variables	**HR**	**95% CI**	***p* Value**	**HR**	**95% CI**	***p* Value**	**HR**	**95% CI**	***p* Value**	**HR**	**95% CI**	***p* Value**
Microenvironment phenotype (Hot versus Cold)	0.31	0.08–1.23	0.09	1.78	0.47–6.70	0.40	0.31	0.07–1.51	0.15	1.82	0.40–8.22	0.44
Age (Continuous)	1.01	0.96–1.06	0.75	0.97	0.89–1.05	0.43	0.96	0.91–1.02	0.23	1.07	0.98–1.17	0.12
T stage (pT2-3 versus pT1)	0.92	0.28–3.08	0.89	2.42	0.58–10.08	0.23	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Nodal status (Positive versus Negative)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	2.50	0.62–10.00	0.20	6.24	1.10–35.34	0.04
Histological grade (Grade I-II versus Grade III)	0.66	0.20–2.18	0.49	0.62	0.15–2.50	0.50	0.65	0.20–2.10	0.47	0.86	0.16–4.70	0.86
Chemotherapy regimen (PCb versus CEF-T)	0.78	0.24–2.50	0.67	0.49	0.11–2.17	0.35	1.02	0.32–3.23	0.97	0.55	0.13–2.44	0.44
Adjuvant radiation (Yes versus No)	0.45	0.14–1.47	0.19	1.30	0.13–13.53	0.83	0.46	0.10–2.03	0.30	0.27	0.04–1.20	0.20

CEF-T, fluorouracil, epirubicin, and cyclophosphamide followed by docetaxel; CI, confidence interval; HR, hazard ratio; N.A., Not appliable; PCb, paclitaxel and carboplatin.

stage pT1 (P = 0.37). Similarly, in the patients with tumor in stage pT2-3, patients of the "hot tumor" phenotype had a borderline significantly longer RFS than the patients of the "cold tumor" phenotype (**Figure 2C**, HR = 0.29, 95%CI 0.06–1.30, P = 0.08). No significant difference in RFS was observed between the two microenvironment phenotypes in the patients with tumors in stage pT1 (HR = 1.76, 95% CI 0.47–6.57, P = 0.39).

To further validate the reliability of the prognostic effect of the microenvironment phenotypes in TNBC, we conducted a multivariate analysis in patients of different nodal statuses and pathologic tumor sizes (**Table 3**). Prognostic relevance of the TNBC microenvironment phenotypes in patients with different status of age, histological grades, and adjuvant radiation therapy was also analyzed. The detail of the results was included in the **Supplementary Contents**.

Microenvironment Phenotypes Relating to the Efficacy of Adjuvant Chemotherapy

Considering the different features of genomic alteration of the microenvironment phenotypes in TNBC (Xiao et al., 2019), we further explored the association between the microenvironment phenotypes and the efficacy of adjuvant chemotherapy regimens. In the patients with "cold tumor", the distribution of RFS was similar in the PCb cohort and the CEF-T cohort (**Figure 3A**, HR = 1.05, 95% CI 0.39–2.82, P = 0.92). In the patients with "hot tumor", no significant difference in RFS was detected between the PCb cohort and the CEF-T cohort (**Figure 3B**, HR = 0.39, 95% CI 0.08–2.01, P = 0.24). Consistently, there was no significant difference in RFS between the patients with "hot tumor" and the patients with "cold tumor" within the PCb arm (HR = 0.39, 95% CI 0.08–1.88, P = 0.22) or the CEF-T arm (HR = 0.99, 95% CI 0.33–2.95, P = 0.98).

Given the prognostic effect of the microenvironment phenotypes in the patients with lymph node metastasis or tumors in stage pT2-3, we examined the association of the microenvironment phenotypes with adjuvant chemotherapy efficacy in these patients who had a relatively higher risk of relapse or metastasis. In the node-positive patients, no significant difference in RFS was observed between the PCb arm and the CEF-T arm no matter in the "hot tumor" subtype (HR = 2.13, 95% CI 0.19–23.63, P = 0.53) or in the "cold tumor" subtype (HR = 0.74, 95% CI 0.21–2.61, P = 0.63). A similar trend of the "cold tumor" phenotype (HR = 1.21 95% CI 0.39–3.75, P = 0.74) was also observed in the patients with tumor in stage pT2-3. RFS events in the "hot tumor" phenotype in patients with tumor in stage pT2-3 were not enough to calculate the HR and 95% CI.

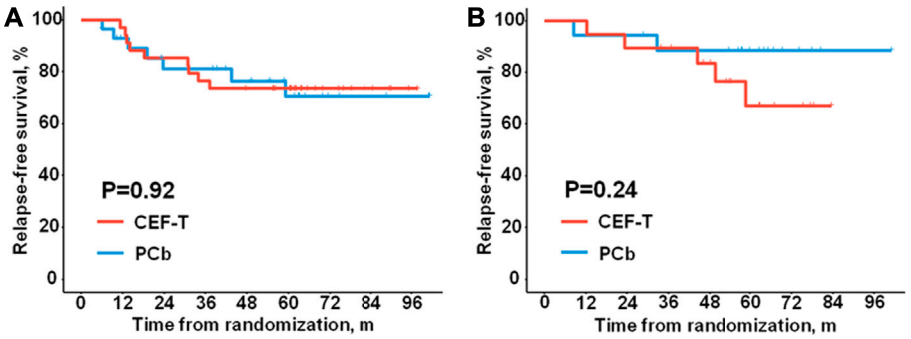

FIGURE 3 | Relapse-free survival of different treatment cohorts. Kaplan-Meier plots show relapse-free survival of **(A)** the patients with "cold tumor", **(B)** the patients with "hot tumor". CEF-T indicates fluorouracil, epirubicin, and cyclophosphamide, followed by docetaxel; PCb, paclitaxel and carboplatin.

DISCUSSION

Taking advantage of the expression data of the early-stage TNBC patients from the PATTERN cohort, we investigated the prognostic significance of the microenvironment phenotype in TNBC and its association with the efficacy of adjuvant chemotherapy regimens.

Recent researches have demonstrated that different cell types in the TME are associated with response to treatment and long-term prognosis of TNBC (Denkert et al., 2010; Su et al., 2014; Denkert et al., 2015; Costa et al., 2018). In our study, we found no significant difference in RFS between the patients of the "hot tumor" phenotype and the patients of the "cold tumor" phenotype. However, in the node-positive patients enrolled in the analysis, the "hot tumor" subtype was related to a significantly better RFS compared with the "cold tumor" subtype, while the distribution of survival of the two subtypes was similar in the node-negative patients. This indicates that the "hot tumor" microenvironment phenotype with abundant adaptive and innate immune cells infiltration might be associated with a better outcome for TNBC patients with lymph node metastasis. Consistently, a borderline significantly longer RFS was observed in the "hot tumor" subtype in the patients with tumor in stage pT2-3, suggesting the prognostic effect of the microenvironment phenotypes in the patients with relatively higher tumor burden. By examining the microenvironment phenotypes in TNBC, we can better distinguish the risk of recurrence and metastasis in node-positive patients and high-risk node-negative patients. Yet, no conclusions could be drawn before further validation is conducted in the prospective study.

In addition, as the microenvironment phenotypes in TNBC have a different level of mutation load and homologous recombination deficiency (Xiao et al., 2019), we subsequently explored its association with the efficacy of different adjuvant chemotherapy for TNBC. No significant difference in RFS was observed between the patients treated by PCb and the patients treated by CEF-T in either of the two phenotypes. There was also no significant difference in RFS between the PCb cohort and the CEF-T cohort in the node-positive patients or stage pT2-3 patients of the two phenotypes. It reflects the limited power of the microenvironment phenotypes in TNBC in predicting the efficacy of a carboplatin-containing regimen.

Our research has some limitations. Firstly, the results presented here are limited by their retrospective character despite using a prospective cohort. Secondly, the limited number of cases with microenvironment phenotype data led to the insufficient statistical power of some tests involved. In addition, CEF-T is no longer a standard recommendation in the National Comprehensive Cancer Network guidelines. At present, epirubicin and cyclophosphamide followed by weekly paclitaxel (EC-wP) might be the optimal choice for TNBC (Sparano et al., 2008). Moreover, considering the limited number of cases enrolled in the analysis, larger prospective studies are necessary to determine whether carboplatin can benefit TNBC patients of certain microenvironment phenotypes.

In conclusion, our study reveals that the microenvironment phenotypes in TNBC might predict the prognosis of the node-positive patients and the high-risk node-negative patients. The association of the microenvironment phenotypes with the efficacy of adjuvant chemotherapy for TNBC remains to be further studied.

DATA AVAILABILITY STATEMENT

All data can be viewed in The National Omics Data Encyclopedia (http://www.biosino.org/node) by pasting the accession (OEP000155) into the text search box or through the URL: http://www.biosino.org/node/project/detail/OEP000155. The HTA 2.0 microarray data is also available in GSE76250 and the RNA sequencing data is available in SRP157974. The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Independent institutional review board of Fudan University Shanghai Cancer Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

This study was Supported by grants from the National Natural Science Foundation of China (grants 81672600, 81722032, and 82072916), the 2018 Shanghai Youth Excellent Academic Leader, the Fudan ZHUOSHI Project, and Chinese Young Breast Experts Research project (CYBER-2021-A01).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2021.752154/full#supplementary-material.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer (K.W.) declared a shared affiliation, with no collaboration, with the authors to the handling editor at the time of the review.

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frontiers in Molecular Biosciences

ORIGINAL RESEARCH
published: 26 October 2021
doi: 10.3389/fmolb.2021.716735

Discovery of Novel Inhibitors From Medicinal Plants for V-Domain Ig Suppressor of T-Cell Activation

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V-domain Ig suppressor of T cell activation (VISTA) is an immune checkpoint and is a type I transmembrane protein. VISTA is linked to immunotherapy resistance, and it is a potential immune therapeutic target, especially for triple-negative breast cancer. It expresses at a high concentration in regulatory T cells and myeloid-derived suppressor cells, and its functional blockade is found to delay tumor growth. A useful medicinal plant database for drug designing (MPD3), which is a collection of phytochemicals from diverse plant families, was employed in virtual screening against VISTA to prioritize natural inhibitors against VISTA. Three compounds, Paratocarpin K (PubChem ID: 14187087), 3-(1H-Indol-3-yl)-2-(trimethylazaniumyl)propanoate (PubChem ID: 3861164), and 2-[(5-Benzyl-4-ethyl-1,2,4-triazol-3-yl)sulfanylmethyl]-5-methyl-1,3,4-oxadiazole (PubChem ID: 6494266), having binding energies stronger than –6 kcal/mol were found to have two common hydrogen bond interactions with VISTA active site residues: Arg54 and Arg127. The dynamics of the compound–VISTA complexes were further explored to infer binding stability of the systems. Results revealed that the compound 14187087 and 6494266 systems are highly stable with an average RMSD of 1.31 Å. Further affirmation on the results was achieved by running MM-GBSA on the MD simulation trajectories, which re-ranked 14187087 as the top-binder with a net binding energy value of –33.33 kcal/mol. In conclusion, the present study successfully predicted natural compounds that have the potential to block the function of VISTA and therefore can be utilized further in experimental studies to validate their real anti-VISTA activity.

Keywords: VISTA, breast cancer, medicinal plant, phytochemical, MD simulation

OPEN ACCESS

Edited by:
Dongqing Wei,
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Reviewed by:
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Specialty section:
This article was submitted to Molecular Diagnostics and Therapeutics, a section of the journal Frontiers in Molecular Biosciences

Received: *29 May 2021*
Accepted: *20 September 2021*
Published: *26 October 2021*

Citation:
Muneer I, Ahmad S, Naz A, Abbasi SW, Alblihy A, Aloliqi AA, Aba Alkhayl FF, Alrumaihi F, Ahmad S, El Bakri Y and Tahir Ul Qamar M (2021) Discovery of Novel Inhibitors From Medicinal Plants for V-Domain Ig Suppressor of T-Cell Activation. Front. Mol. Biosci. 8:716735. doi: 10.3389/fmolb.2021.716735

INTRODUCTION

Immunotherapy has turned into an important pillar of cancer treatment due to the successful blocking of the programmed cell death protein 1 (PD-1) and its ligand-programmed death-ligand 1 (PD-L1) immune checkpoints. Immune checkpoint receptors control the duration and intensity of immune response by inhibiting T cell activation (Tang et al., 2018). Several immune checkpoint proteins have been discovered, such as PD-1/PD-L1, TIGIT, VISTA, cytotoxic T lymphocyte antigen-4 (CTLA-4), TIM3, BTLA, and LAG3. PD-1 inhibitors such as nivolumab, pembrolizumab, and cemiplimab and the human IgG1 k anti-CTLA-4 monoclonal antibody ipilimumab have been approved by the Food and Drug Administration (FDA). These approved drugs have become successful cancer therapies. However, the relatively low response rate of current immunotherapy drugs (less than 30%) is still a serious challenge, and efforts are needed to identify and overcome other immunosuppressive pathways (Ventola, 2017).

In the ever-expanding list of immune checkpoints, VISTA (V-domain immunoglobulin suppressor of T cell activation) is considered to be an important regulator of the immune system. VISTA immune checkpoint protein is a type 1 transmembrane protein that is encoded by the C10orf54 gene (Wang et al., 2011). VISTA is part of the B7 family consisting of a single extracellular N-terminal Ig-V domain, a stalk with approximately 30 amino acids, a transmembrane domain, and a cytoplasmic domain (Flies et al., 2011). The closest homolog of VISTA in the B7 family is PD-L1, sharing 23% sequence identity. VISTA is highly expressed in tumor-infiltrating lymphocytes. VISTA is also expressed in $CD4^+$ and $CD8^+$ cells, where it negatively regulates T cell responses (Borggrewe et al., 2018; ElTanbouly et al., 2019). It has also been observed that VISTA is highly expressed in breast cancer as compared to other cancer types, indicating that targeting VISTA may benefit breast cancer immunotherapy (Xie et al., 2020). Interestingly, expression of VISTA has also been observed in different cancer types such as breast invasive carcinoma (BRCA), invasive ductal carcinoma (IDC), bladder urothelial carcinoma (BLCA), colon adenocarcinoma (COAD), kidney chromophobe (KICH), lung squamous cell carcinoma (LUSC), uterine carcinosarcoma (UCS), and skin cutaneous melanoma (SKCM). Recently, it has been reported that VISTA is the acidic pH selective ligand of PSGL-1, which means that it may engender resistance to antitumor immune response (Johnston et al., 2019). Research on a variety of clinical samples, autoimmune disease models, and tumor models has shown that VISTA has a key regulatory effect on the immune system and has the potential to be used as a therapeutic or combined drug target. These findings indicated that the high expression of VISTA on tumor cells in about 20% of NSCLC specimens can prove the feasibility of targeting VISTA for cancer therapy (Cuzick et al., 2015). Clinical studies have shown that the expression of VISTA is upregulated in oral squamous cell carcinoma and gastric cancer (Böger et al., 2017; Wu et al., 2017). After ipilimumab therapy, the VISTA immune checkpoint has also increased in patients with prostate cancer (Gao et al., 2017; Kakavand et al., 2017). In addition, previous studies have shown that VISTA is highly expressed in the immune cell subsets of human pancreatic cancer patients (Xie et al., 2018b).

Currently, compound CA-170 is undergoing phase I clinical trial for advanced tumors and lymphoma. CA-170 exhibits powerful activity to stop the lymphocyte proliferation and effector functions inhibited by VISTA proteins. CA-170 also exhibits selectivity for other immune checkpoints such as CTLA4, BTLA, and LAG3. These nonclinical data provide a strong basis for the clinical development of CA-170 (Sasikumar and Ramachandra, 2018; Wang et al., 2018; Blevins et al., 2019). In this study, we performed molecular docking to select natural drugable molecules from medicinal plants which may act as antagonists against VISTA. Molecular dynamics (MD) studies were carried out to further verify the binding of natural leads with VISTA protein.

MATERIALS AND METHODS

Phytochemical's Library Retrieval and Filtration

The MPD3 database's (https://www.bioinformation.info/) (Mumtaz et al., 2017) diverse and ready-to-dock library of phytochemicals was retrieved and filtered for lead-like molecules. Lead molecules may serve as the starting point for further structural optimization and have the best chance to become good drug candidates. The lead molecule filtration was accomplished through the online FAF-Drugs4 server (Lagorce et al., 2017). The different physicochemical parameters applied during filtration include the following: molecular weight (150–400 kDa), logP (−3 to 4), hydrogen bond donor number (≤4), hydrogen bond acceptor number (≤7), TPSA (≤160), rotatable bonds (≤9), rigid bonds (≤30), rings (≤4), maximum ring size of system (≤18), number of heteroatoms (1–15), carbon number (3–35), charges (≤4), ratio of H/C (0.1–1.1), total charge (−4 to 4), and stero centers (≤2). These parameters were applied in accordance with Lipinski's (Lipinski et al., 1997), Veber's (Veber et al., 2002), and Egan's (Egan et al., 2000) rules, to screen out the most promising hits for downward analysis.

Docking Studies

The human VISTA extracellular domain crystal structure present in the RCSB PDB database with the PDB ID: 6OIL was retrieved and processed in UCSF Chimera (Pettersen et al., 2004) for the molecular docking process. The structure was prepared first by removing co-crystalized water molecules and the NAG ligand, and then missing hydrogen atoms were added and minimized for energy *via* two algorithms, conjugate gradient and steepest descent, keeping the step size of 0.02 Å. Autodock Vina (Trott and Olson, 2010) was used to dock the ligand library against VISTA. We set the number of binding modes to 20 and exhaustiveness to 20. The grid dimensions were 40 × 40 × 40 (x, y, z), focused on the binding site of the VISTA native ligand along the XYZ dimension of 28.474 × 31.645 × 34.012 Å. Each docked pose was ranked using the Vina empirical scoring function where the most negative binding energy implies the

most stable complex. The top 20 docked ligands with the lowest docking energy were considered for further analyses. Protein–ligand interactions were visualized using Pymol (DeLano, 2002).

Analysis of Complex Dynamics Using MD Simulations

The FF14SB force field of the AMBER 18 (Case et al., 2012) molecular dynamics (MD) simulation package was used for preparation of protein parameters, while its GAFF force field was used for generating ligand parameters (Wang et al., 2004). The whole system was solvated in the water box (TIP3P), considering the padding distance of 12 Å (Jorgensen et al., 1983). Particle mesh Ewald (PME) was employed for processing long-range electrostatic interactions (Darden et al., 1993), and for the nonbonded interactions, the distance cutoff was allowed to be 10 Å. The SHAKE algorithm was used to constrain the bonds involving hydrogen (Ryckaert et al., 1977). All the systems were subjected to energy minimization by running 1,000 steps of the steepest descent and conjugate gradient algorithms. Temperature of each system was equilibrated to 300 K using NVT for a time period of 20 picoseconds (ps), gradually. Afterward, the system equilibration was achieved using NPT ensemble. Finally, a production run of 50 ns was performed, and each trajectory was saved after every 2 fs. Root mean square deviation (RMSD) and root mean square fluctuation (RMSF) analysis of all trajectories was performed to check the system stability by using module CPPTRAJ (Roe and Cheatham, 2013).

Free Energies Estimation by AMBER MMPBSA.py

The MM-GBSA method in AMBER 18 was used to estimate free energies binding for complexes (Miller et al., 2012). 100 snapshots separated at equal intervals were collected from MD trajectories to carry out the binding free energy calculations. In MM-GBSA, estimation of the net binding free energy (ΔG_{bind}) is done as follows:

$$\Delta G_{binding} = \Delta G_{complex\ (receptor+ligand)} - \left(\Delta G_{receptor} + \Delta G_{ligand}\right). \quad (1)$$

In **Equation 1**, $\Delta G_{complex}$ is complex free energy, $\Delta G_{receptor}$ is receptor free energy, and ΔG_{ligand} is ligand free energy. The free energy of the above terms can be gained by using the equations given below:

$$\Delta G = \Delta G_{gas} + \Delta G_{sol} - T\Delta S_s \quad (2)$$

$$\Delta G_{gas} = \Delta E_{elec} + \Delta E_{vdw}. \quad (3)$$

$$\Delta G_{sol} = \Delta G_{GB} + \Delta G_{SA}. \quad (4)$$

In **Equation 2**, ΔG is the free energy. $T\Delta S$ corresponds to entropy energy. In **Eq. 3**, the electrostatic interaction energy (ΔE_{elec}) and van der Waals interaction energy (ΔE_{vdw}) collectively correspond to the molecular mechanics energy in the gas phase (ΔG_{gas}). The polar contribution (ΔG_{GB}) and the nonpolar contribution (ΔG_{SA}) result in solvation free energy (ΔG_{sol}). The MM-PBSA method takes more time than MM-GBSA. Hou T et al. described that to calculate the relative ΔG_{bind}, MM-GBSA is better in terms of result accuracy than MM-PBSA (Gohlke et al., 2004; Hou et al., 2011; JyrkkäRinne et al., 2012). This approach has been extensively employed in protein–protein interaction and protein–ligand binding studies (Alamri et al., 2021; Tahir ul Qamar et al., 2021).

Computational Prediction of Compound Pharmacokinetics

The selected compounds were also subjected to different predictions such as drug-likeness, lead-likeness, pharmacokinetics, medicinal chemistry, and toxicity to guide synthetic chemists in optimizing the structure to be successful in clinical studies. Computational predictions of the compound parameters as discussed above were done using the SWISSADME server (Daina et al., 2017).

RESULTS AND DISCUSSION

Retrieval of Lead Compounds From MPD3 Database

The proposed research involves virtual screening of the MPD3 database against VISTA protein, followed by MD simulations and MM-GBSA methods. MPD3 is a collection of uniquely retrieved phytochemical compounds with reported therapeutic potential. The natural compounds were preferred because they are safer, possess better pharmacokinetics, and are easy to test in further experimental studies (Riaz et al., 2017). The lead-like compounds from MPD3 were considered to be therapeutically useful in the drug discovery process, as such compounds have improved selectivity, potency, and medicinal chemistry parameters (Hughes et al., 2011). Additionally, such compounds' structures can be easily optimized to get the desired biological activity. Previously, only Li et al. (2020) and Gabr and Gambhir (2020) reported small-molecule inhibitors against VISTA. Thus, lead-like natural compounds from MPD3 were retrieved (**Figure 1**). In total, 1,634 molecules were able to fulfill the criteria of lead-like compounds. Theses 1,634 compounds were used for subsequent docking studies.

Molecular Docking of CA-170 Into VISTA Immune Checkpoint

The CA-170 small molecule has been reported as a dual inhibitor of PDL1/L2 and VISTA in order to treat advanced solid tumors and lymphomas. CA-170 is under phase II clinical trials for head and neck/oral cavity cancer, MSI-H positive cancers, lung cancer, and Hodgkin lymphoma in India. Its exact chemical structure has not been disclosed; however, some studies suggested that CA-170 is a peptidomimetic compound, composed of D-asparagine, L-serine, and L-threonine (Sasikumar et al., 2018; Musielak et al., 2019). Recently, the X-ray structure of the human VISTA extracellular domain has been solved at a resolution of 1.85 Å (PDB ID: 6OIL).

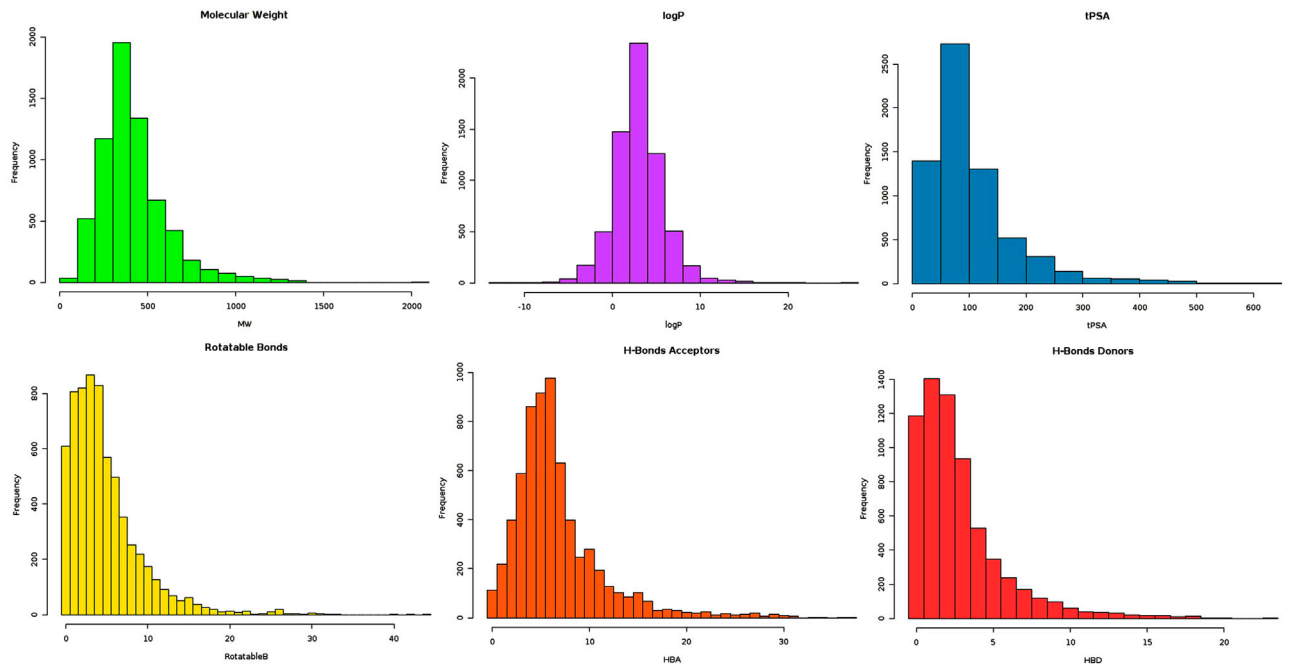

FIGURE 1 | Graphical illustration of FAF-Drugs4 server output, highlighting distribution of lead-like compounds on different parameters (bars of different colors are associated with different parameter results).

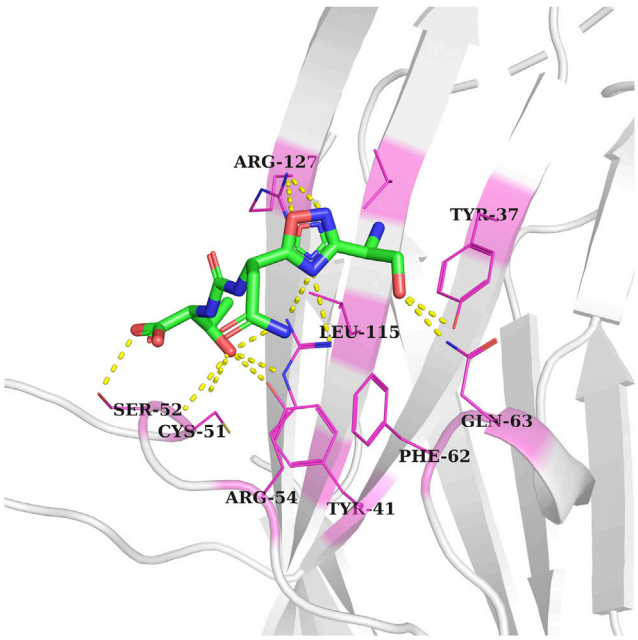

FIGURE 2 | Docked conformation of the CA-170 inhibitor in the VISTA active pocket (gray color). Interacting residue of VISTA is shown as pink-colored lines-cartoons and labeled. Meanwhile, hydrogen bonding between residues is represented by yellow-colored dotted lines.

VISTA is implicated in different cancers, including breast (Zong et al., 2020), skin (melanoma) (Kakavand et al., 2017), prostate (Gao et al., 2017), colon (Xie et al., 2018a), pancreatic (Xie et al., 2018b), ovarian (Mulati et al., 2019), and lung cancer (Villarroel-Espindola et al., 2018). A single-point mutation study to find the essential residues involved in the interaction of anti-VISTA antibody VSTB showed that three residues, Arg54, Phe62, and Gln63, are essential for the binding of VISTA to VSTB. The latter suggested that targeting these residues would be a valuable approach to inhibiting the VISTA immune checkpoint. In order to predict the binding pocket of CA-170 within the VISTA immune checkpoint, a flexible structure-based docking of CA-170 (PubChem ID: 123843830) using Autodock vina software was performed, following the same protocol as mentioned in the methodology. The grid box which represents the docking search area was centered to cover three key residues (Arg54, Phe62, and Gln63). Interestingly, the top pose of CA-170 with the lowest binding energy was forming hydrogen bonds with the Tyr41, Tyr37, Cys51, Ser52, and Arg127, including two crucial residues, Arg54 and Gln63 (**Figure 2**). Previous study indicated that a single-point mutation of Arg54 into Ala led to the abolition of the binding of anti-VISTA antibody VSTB to VISTA (Mehta et al., 2019). These results validated the docking protocol being applied in this study.

Virtual Screening

The molecular docking approach, one of the reliable approaches in the drug discovery process, was used to determine the natural inhibitors of VISTA protein. Docked ligands were graded based on their binding energy scores. The pose with the lowest score compared to CA-170 was regarded as the stable binding mode of the ligand. The top 20 compounds were visually analyzed using PyMol; out of those 20, three compounds were selected based on the binding conformation and interactions with the active site key

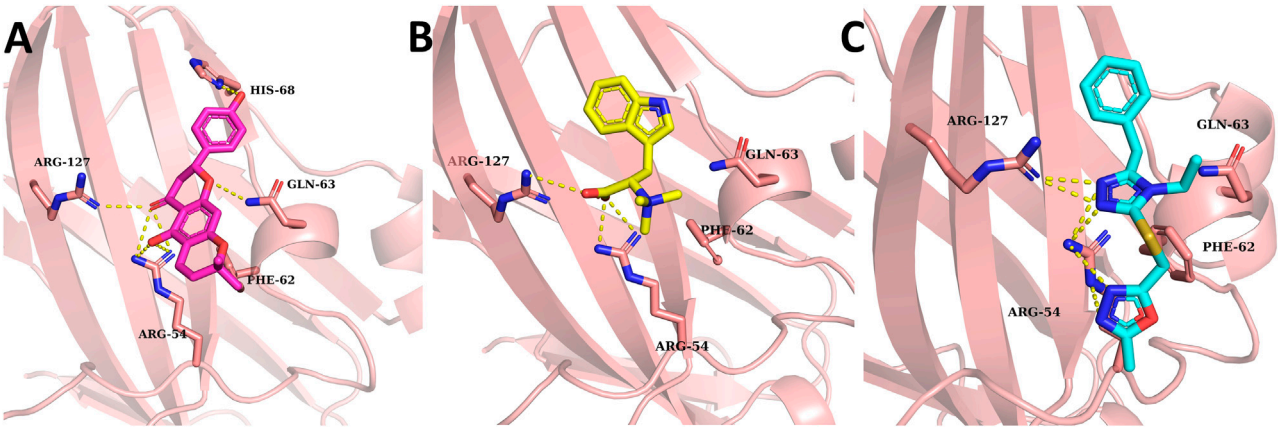

FIGURE 3 | Binding conformation and hydrogen binding residues of the top three compounds at the active site of VISTA (light pink). **(A)** 14187087 (dark pink), **(B)** 3861164 (yellow), and **(C)** 6494266 (cyan). Meanwhile, hydrogen bonding between residues is represented by yellow-colored dotted lines.

TABLE 1 | Filtered best affinity binders of VISTA. The binding affinity score, total number of hydrogen bonds, and VISTA residues involved in hydrogen bonding with the compound.

PubChem ID	Energy score (kcal/mol)	Total number of hydrogen bonds	Interacting residues with hydrogen bonding
14187087	−6.3	7	Arg54, Gln63, His68, Arg127
3861164	−6.8	3	Arg54, Arg127
6494266	−6.7	6	Arg54, Arg127
CA170	−5.4	9	Tyr37, Gln63, Arg127, Arg54, Tyr41

residues. These top selected natural ligands were successfully docked in the target active site. The ligand binding poses are depicted in **Figure 3**.

All the compounds had at least one hydrogen bond with the critical active site residues. Among all three ligands' compounds, 14187087 has a greater number of hydrogen bonds with an energy value of −6.3 kcal/mol. It formed hydrogen bonds with Arg54, Gln63, His68, and Arg127 residues, out of which two residues (Arg54 and Gln63) are important active site residues. Compounds 3861164 and 6494266 formed two hydrogen bonds with Arg54 and Arg127 with the binding scores −6.8 kcal/mol and −6.7 kcal/mol, respectively (**Table 1**). All the ligands have two common interactions with Arg54 and Arg127. As the compounds revealed favorable docking scores and good atomic-level chemical interactions, including hydrogen bonding with the VISTA, dynamics supported by binding free estimation were undertaken to further investigate the applicability of these compounds as effective VISTA inhibitors.

Molecular Dynamics Simulation Analysis of the Docked Complexes

All atom MD simulation was conducted using the AMBER package to assess the validity of the docking data and results by analyzing the dynamics behavior of protein atoms and the stability of the compounds at the binding site. For a time scale of 50 ns, the systems were analyzed for structure stability using RMSD and RMSF. The CPPTRAJ module of AMBER 18 was used to calculate the RMSD values to determine the convergence of the trajectories. RMSF values were calculated to determine the structure flexibilities of protein. Even though docking studies have been used effectively for calculating the ligand binding pose for several proteins, they failed to assess the ligand binding affinity (Cheng et al., 2012). During docking, proteins are treated as rigid molecules which do not consider the conformational changes that occur due to the ligand binding (Heitz and Van Mau, 2002). These conformational changes can be studied using molecular dynamics simulations. MD simulations have been extensively used to study the conformational changes in the protein–ligand interactions (Li et al., 2011).

Compound 6494266 fluctuated up to 3.5 Å during the first 5 ns, but later, after 15 ns, it reached equilibrium. Among all the complexes, the 14187087 compound was the most stable complex throughout the simulation with an average RMSD of 1.31 Å. However, the 3861164 complex kept oscillating throughout the simulation, indicating that this complex might be unstable among all the complexes. Thereafter, the 14187087 and 6494266 complexes were stabilized and showed steady state dynamic behavior, as shown in **Figure 4**.

The variability in the conformation of trajectories can be monitored by calculating RMSF for individual atoms. In order to investigate and explore the conformational variability of each trajectory, RMSF of residues was plotted with respect to the residue number to show the local conformational changes for all the simulated complexes (**Figure 5**). Among all the docked

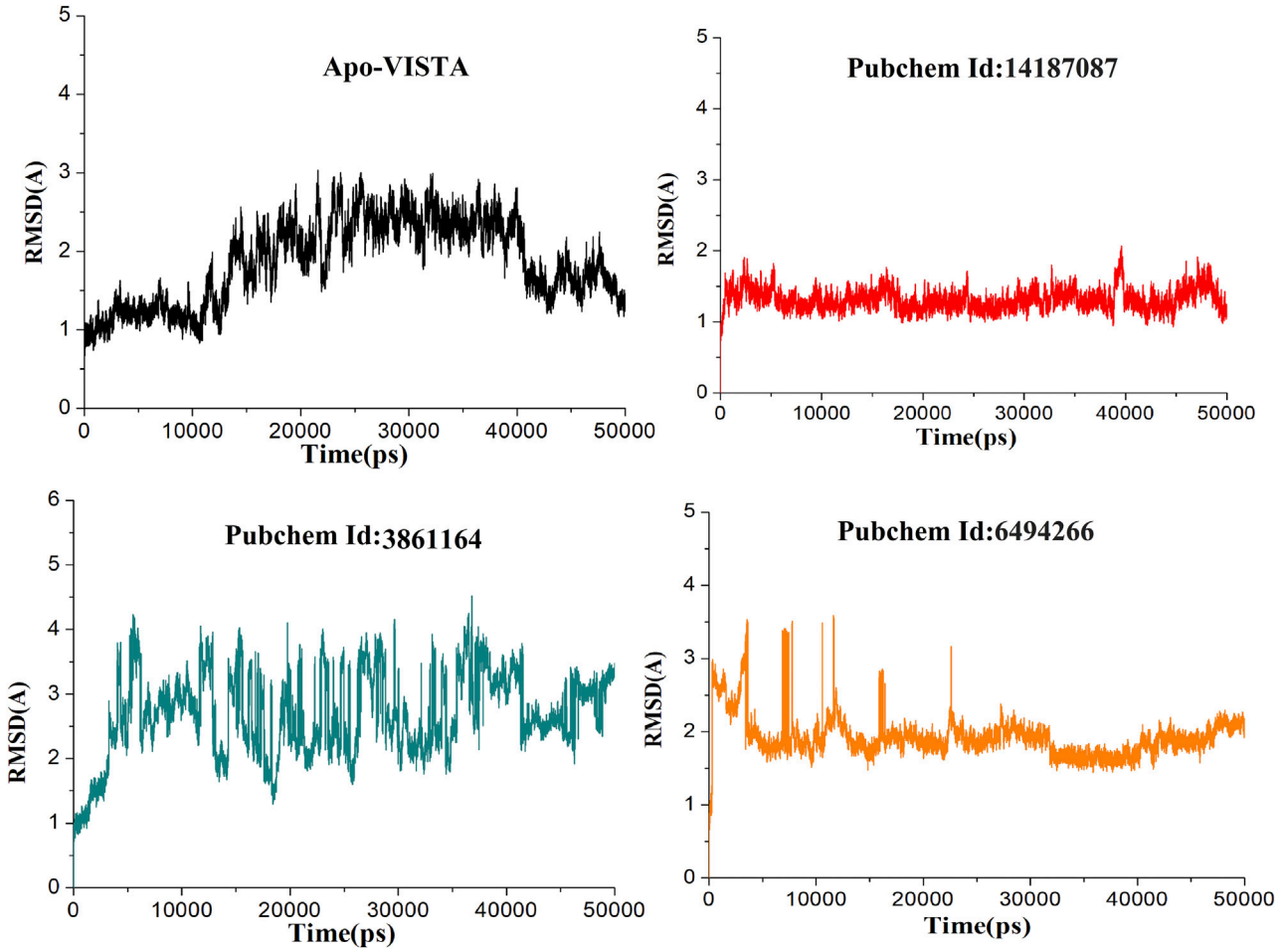

FIGURE 4 | RMSD of all simulated systems as a function of time (Apo-VISAT: black, PubChem ID: 14187087-VISTA: red, PubChem ID: 3861164-VISTA: green, and PubChem ID: 6494266-VISTA: orange).

complexes, compound 3861164 and compound 6494266 showed high fluctuations as compared to other systems, which is also consistent with the RMSD results. It can be concluded that the apo-VISTA structure, despite one large peak (40–52 amino acids), is highly stable compared to the VISTA–compound complexes. Conformational rearrangement of the loops than the rest of the protein in the presence of compounds has been previously reported and is linked to greater flexibility (Streaker and Beckett, 1999; Danielson and Lill, 2012). As VISTA protein has higher loop percentage and has a small size, upon ligand binding it is highly likely that loops may behave more dynamically. However, these fluctuations did not disturb the ligand binding conformation and the chemical interaction network, which are key to the stable binding of the compounds throughout the simulation time.

Analysis of Intermolecular Binding Stability by MM-GBSA

MM-GBSA binding free energies of the complexes were estimated to validate the docking and simulation results. Such MM-GBSA binding free energy is now regularly applied in drug-designing protocols as they are more reliable than conventional docking techniques and less computationally expensive (Alamri et al., 2020a; Alamri et al., 2020b). The binding energies of complexes are presented in **Table 2**. It was observed that van der Waals energy and electrostatic energy dominated chemical interactions between the compounds and VISTA protein and contributed majorly to the total energy. In the interaction of 14187087, the van der Waals and electrostatic energy values were −32.2723 and −49.3294 kcal/mol, respectively, suggesting that electrostatic interactions were the major forces in the binding of VISTA and compound-1. In the case of 3861164, the contribution of van der Waals energy was −21.4642 kcal/mol and that of electrostatic energy was −16.8891 kcal/mol. In the case of complex 6494266, van der Waals and electrostatic energy was −27.7207 and −37.0189 kcal/mol, respectively. Among all three complexes, 14187087 had the minimum binding energy, indicating it as an effective inhibitor.

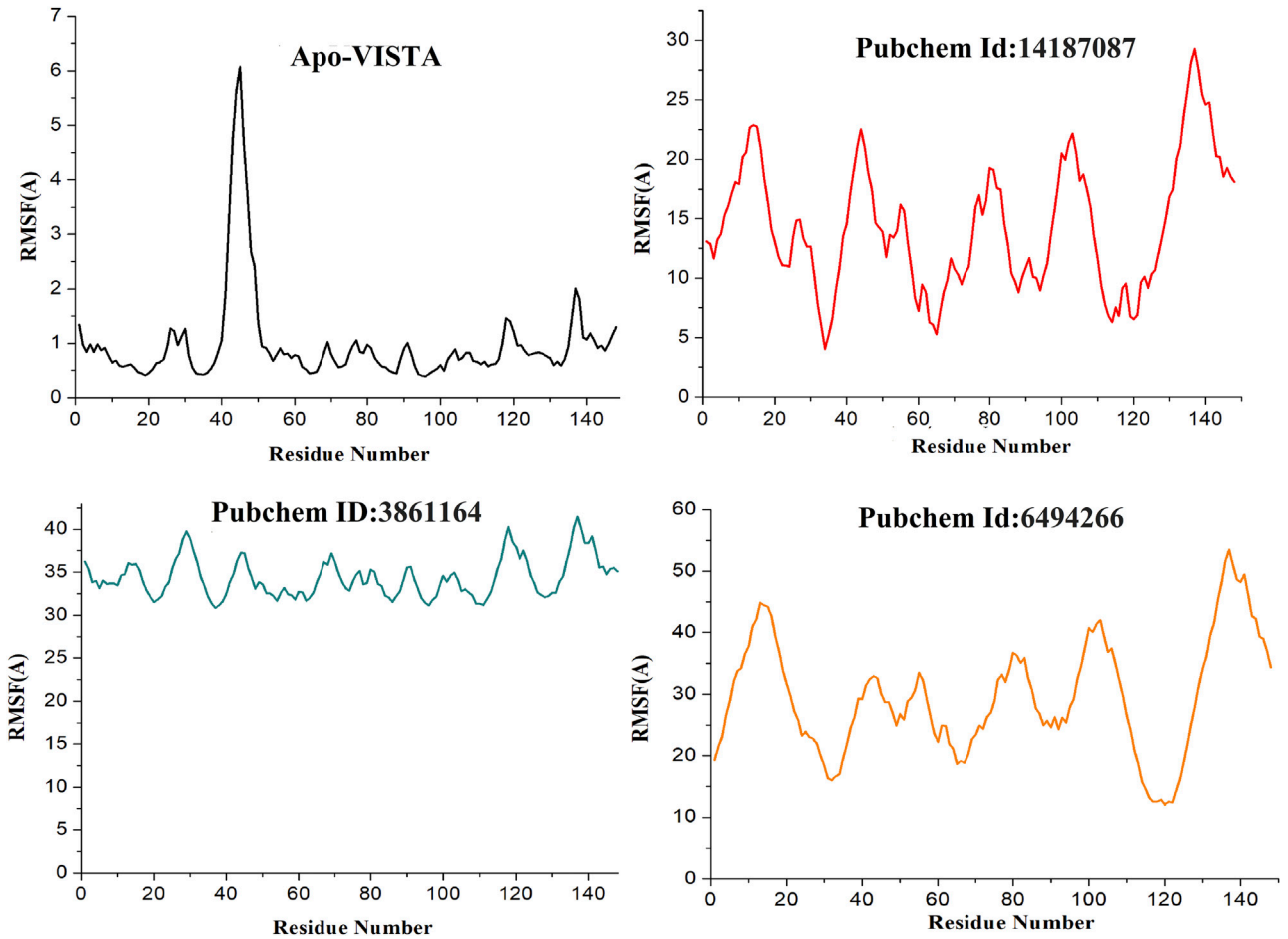

FIGURE 5 | RMSF of simulated systems. The protein residues (on the *x*-axis) are plotted versus their flexibility (on the *y*-axis) from the mean position in simulation time (Apo-VISAT: black, PubChem ID: 14187087-VISTA: red, PubChem ID: 3861164-VISTA: green, and PubChem ID: 6494266-VISTA: orange).

TABLE 2 | Binding free energy calculations of all three complexes. ΔEgas, molecular mechanics energy in the gas phase; ΔEele, electrostatic energy; ΔEvdw, van der Waals potential energy; ΔGsol, solvation free energy; ΔGbind, binding energy.

Energy kcal/mol	14187087	3861164	6494266
ΔEvdw	−32.27 ± 4.50	−21.46 ± 6.16	−27.72 ± 3.30
ΔEele	−49.33 ± 14.30	−16.88 ± 9.93	−37.01 ± 11.80
ΔEgas	−81.60 ± 16.75	−38.35 ± 14.14	−64.74 ± 11.76
ΔGsol	48.27 ± 9.01	24.68 ± 9.14	43.76 ± 9.08
ΔGbind	−33.33 ± 9.97	−13.67 ± 6.38	−20.97 ± 4.18

Computational Prediction of Compound Pharmacokinetics

SwissADME is an online server for calculating different physical and chemical indicators and predicting drug-like properties, ADME parameters, pharmacochemical friendliness, and pharmacokinetic properties to help drug discovery. Detailed results of all the compounds are listed in **Table 3**. The oral bioavailability radar of the compounds is shown in **Figure 6**.

The physicochemical properties of the compounds are within the scope of drug-likeness and do not violate any Lipinski rule parameter. In addition, the compounds have good lipophilicity, so they can be transported to the maximum extent and reach the target site (Arnott and Planey, 2012). The compounds were also demonstrated to fulfill all requirements of the prominent Lipinski (Lipinski, 2004), Egan (Egan et al., 2000), Muegge (Muegge et al., 2001), and Veber (Veber et al., 2002) drug-ability rules. The compounds were predicted to be soluble and thus can be good candidates for oral administration. All the compounds were also predicted to not contain pan-assay interference compounds (PAINS) alerts and will not interact nonspecifically with multiple biological targets. This analysis revealed that the screened hits are VISTA-specific and will not have off-target effects. The compounds also have good gastrointestinal absorption, thus indicating that the good concentration of the drugs can reach the target site for performing the required action. Also, the compounds have good synthetic accessibility scores; therefore, they are easy to synthesize for experimental studies.

TABLE 3 | Overview of different physicochemical properties, pharmacokinetics, medicinal chemistry, and drug-likeness of the compounds.

Physicochemical properties		Pharmacokinetics	
PubChem ID: 14187087			
Formula	C20H18O5	GI absorption	High
Molecular weight	338.35 g/mol	BBB permeant	Yes
Number of heavy atoms	25	P-gp substrate	Yes
Number of arom. heavy atoms	12	CYP1A2 inhibitor	Yes
Fraction Csp3	0.25	CYP2C19 inhibitor	Yes
Number of rotatable bonds	1	CYP2C9 inhibitor	Yes
Number of H-bond acceptors	5	CYP2D6 inhibitor	Yes
Number of H-bond donors	2	CYP3A4 inhibitor	Yes
Molar refractivity	93.67	Log K_p (skin permeation)	–5.76 cm/s
TPSA	75.99 Å^2		
Lipophilicity		Drug-likeness	
Log $P_{o/w}$ (iLOGP)	2.97	Lipinski	Yes; 0 violation
Log $P_{o/w}$ (XLOGP3)	3.67	Ghose	Yes
Log $P_{o/w}$ (WLOGP)	3.56	Veber	Yes
Log $P_{o/w}$ (MLOGP)	1.82	Egan	Yes
Log $P_{o/w}$ (SILICOS-IT)	3.44	Muegge	Yes
Consensus log $P_{o/w}$	3.09	Bioavailability score	0.55
Water solubility		Medicinal chemistry	
Log *S* (ESOL)	–4.54	PAINS	0 alert
Solubility	9.78e-03 mg/ml; 2.89e-05 mol/L	Brenk	1 alert: quaternary_nitrogen_2
Class	Moderately soluble	Lead-likeness	No; 1 violation: XLOGP3>3.5
Log *S* (Ali)	–4.96	Synthetic accessibility	3.97
Solubility	3.75e-03 mg/ml; 1.11e-05 mol/L		
Class	Moderately soluble		
Log *S* (SILICOS-IT)	–4.83		
Solubility	5.04e-03 mg/ml; 1.49e-05 mol/L		
Class	Moderately soluble		
PubChem ID: 3861164			
Formula	C14H18N2O2	GI absorption	High
Molecular weight	246.30 g/mol	BBB permeant	No
Number of heavy atoms	18	P-gp substrate	Yes
Number of arom. heavy atoms	9	CYP1A2 inhibitor	No
Fraction Csp3	0.36	CYP2C19 inhibitor	No
Number of rotatable bonds	4	CYP2C9 inhibitor	No
Number of H-bond acceptors	2	CYP2D6 inhibitor	No
Number of H-bond donors	1	CYP3A4 inhibitor	No
Molar refractivity	69.50	Log K_p (skin permeation)	–6.23 cm/s
TPSA	55.92 Å^2		
Lipophilicity		Drug-likeness	
Log $P_{o/w}$ (iLOGP)	–1.65	Lipinski	Yes; 0 violation
Log $P_{o/w}$ (XLOGP3)	2.21	Ghose	Yes
Log $P_{o/w}$ (WLOGP)	0.54	Veber	Yes
Log $P_{o/w}$ (MLOGP)	–2.31	Egan	Yes
Log $P_{o/w}$ (SILICOS-IT)	1.94	Muegge	Yes
Consensus log $P_{o/w}$	0.14	Bioavailability score	0.55
Water solubility		Medicinal chemistry	
Log *S* (ESOL)	–2.87	PAINS	0 alert
Solubility	3.36e-01 mg/ml; 1.36e-03 mol/L	Brenk	1 alert: quaternary_nitrogen_2
Class	Soluble	Lead-likeness	No; 1 violation: MW < 250
Log *S* (Ali)	–3.02	Synthetic accessibility	2.41
Solubility	2.36e-01 mg/ml; 9.58e-04 mol/L		
Class	Soluble		
Log *S* (SILICOS-IT)	–4.33		
Solubility	1.14e-02 mg/ml; 4.65e-05 mol/L		
Class	Moderately soluble		
PubChem ID:6494266			
Formula	C15H17N5OS	GI absorption	High
Molecular weight	315.39 g/mol	BBB permeant	No
Number of heavy atoms	22	P-gp substrate	Yes

(Continued on following page)

TABLE 3 | (*Continued*) Overview of different physicochemical properties, pharmacokinetics, medicinal chemistry, and drug-likeness of the compounds.

Physicochemical properties		Pharmacokinetics	
Number of arom. heavy atoms	16	CYP1A2 inhibitor	Yes
Fraction Csp3	0.33	CYP2C19 inhibitor	Yes
Number of rotatable bonds	6	CYP2C9 inhibitor	Yes
Number of H-bond acceptors	5	CYP2D6 inhibitor	No
Number of H-bond donors	0	CYP3A4 inhibitor	Yes
Molar refractivity	84.57	Log K_p (skin permeation)	–6.60 cm/s
TPSA	94.93 Å^2		
Lipophilicity		Drug-likeness	
Log $P_{o/w}$ (iLOGP)	2.97	Lipinski	Yes; 0 violation
Log $P_{o/w}$ (XLOGP3)	2.29	Ghose	Yes
Log $P_{o/w}$ (WLOGP)	2.72	Veber	Yes
Log $P_{o/w}$ (MLOGP)	1.86	Egan	Yes
Log $P_{o/w}$ (SILICOS-IT)	2.94	Muegge	Yes
Consensus log $P_{o/w}$	2.52	Bioavailability score	0.55
Water solubility		Medicinal chemistry	
Log *S* (ESOL)	–3.38	PAINS	0 alert
Solubility	mg/ml; 4.17e-04 mol/l	Brenk	0 alert
Class	Soluble	Lead-likeness	
			Yes
Log *S* (Ali)	–3.92	Synthetic accessibility	3.24
Solubility	3.78e-02 mg/ml; 1.20e-04 mol/l		
Class	Soluble		
Log *S* (SILICOS-IT)	–5.62		
Solubility	7.53e-04 mg/ml; 2.39e-06 mol/l		
Class	Moderately soluble		

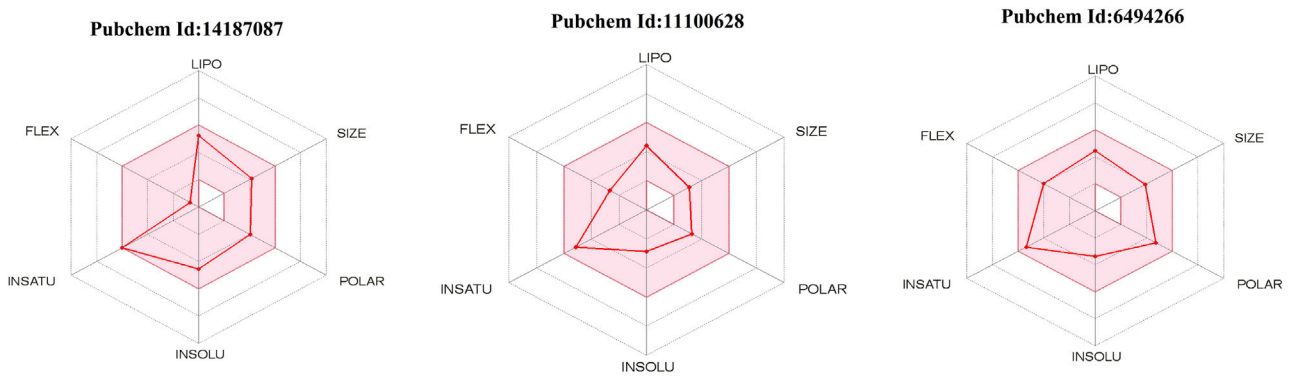

FIGURE 6 | Radar of oral bioavailability (shown by red line). The pink-colored zone presents the physicochemical space allowed for orally bioavailable drugs. INSATU (instauration), INSOLU (insolubility), LIPO (lipophility), POLAR (polarity), FLEX (flexibility), and SIZE (molecular weight).

CONCLUSION

The study short-listed Paratocarpin K (PubChem ID: 14187087), 3-(1H-Indol-3-yl)-2-(trimethylazaniumyl)propanoate (PubChem ID: 3861164), and 2-[(5-Benzyl-4-ethyl-1,2,4-triazol-3-yl) sulfanylmethyl]-5-methyl-1,3,4-oxadiazole (PubChem ID: 6494266) from the MPD3 database as effective natural lead inhibitory molecules against VISTA protein, which is an immune checkpoint protein and is considered as a potential therapeutic target, especially for treating triple-negative breast cancer. These molecules unveiled good binding affinity as predicted by the docking technique and showed stable binding modes at the active pocket of VISTA protein. The compounds' docked conformation dynamics study validated their stable binding nature and compounds remained intact at the active site by both hydrophobic and hydrophilic interactions with key active residues of the protein. Additionally, confirmation on the binding stability of the compounds was accomplished through the binding free energy approach, which also revealed consistent results with the docking and MD simulation outcomes. The study employed a comprehensive computational framework to identify anticancer molecules by targeting VISTA protein. Although each step is thoroughly validated and the results are investigated for accuracy *via* follow-up computational approaches, the study suffers from lack of experimental validation. Altogether, the study findings are promising and could be subjected to further experimental evaluation to disclose their anti-VISTA/cancer potency.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

IM and SaA: conceptualization, data curation, software, methodology, investigation, and writing—original draft preparation. AN, SuA, AdA, AbA, FFA, SFA, and YE: visualization, validation, and writing—reviewing and editing. FA and MT: resources, supervision, project administration, funding acquisition, and writing—reviewing and editing.

ACKNOWLEDGMENTS

Researchers would like to thank the Deanship of Scientific Research, Qassim University, for funding publication of this project.

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frontiers in Molecular Biosciences

REVIEW
published: 25 January 2022
doi: 10.3389/fmolb.2022.836417

Triple-Negative Breast Cancer: A Brief Review About Epidemiology, Risk Factors, Signaling Pathways, Treatment and Role of Artificial Intelligence

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Triple-negative breast cancer (TNBC) is a kind of breast cancer that lacks estrogen, progesterone, and human epidermal growth factor receptor 2. This cancer is responsible for more than 15–20% of all breast cancers and is of particular research interest as it is therapeutically challenging mainly because of its low response to therapeutics and highly invasive nature. The non-availability of specific treatment options for TNBC is usually managed by conventional therapy, which often leads to relapse. The focus of this review is to provide up-to-date information related to TNBC epidemiology, risk factors, metastasis, different signaling pathways, and the pathways that can be blocked, immune suppressive cells of the TNBC microenvironment, current and investigation therapies, prognosis, and the role of artificial intelligence in TNBC diagnosis. The data presented in this paper may be helpful for researchers working in the field to obtain general and particular information to advance the understanding of TNBC and provide suitable disease management in the future.

Keywords: triple negative breast cancer, risk factor, signaling pathways, prognosis, artificial intelligence

OPEN ACCESS

Edited by:
Abbas Khan,
Shanghai Jiao Tong University, China

Reviewed by:
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Specialty section:
This article was submitted to Molecular Diagnostics and Therapeutics, a section of the journal Frontiers in Molecular Biosciences

Received: *15 December 2021*
Accepted: *07 January 2022*
Published: *25 January 2022*

Citation:
Almansour NM (2022) Triple-Negative Breast Cancer: A Brief Review About Epidemiology, Risk Factors, Signaling Pathways, Treatment and Role of Artificial Intelligence. Front. Mol. Biosci. 9:836417. doi: 10.3389/fmolb.2022.836417

INTRODUCTION

Breast cancer is a pathological condition that occurs in the breast tissue. In most cases, emergence occurs from the milk duct, while other minor cases occur from lobules. The cancer of the ductile region is known as ductal carcinoma, while those involving mammary lobules are called lobular carcinomas (Medina et al., 2020; Muneer et al., 2021). According to the World Health Organization (WHO) reports, breast cancer is placed second on the list of common diseases worldwide. Breast cancer has been observed to cause more mortality in the United States and Europe after lung cancer. The disease is also very common in less developed countries (Ghoncheh et al., 2016; Suleman et al., 2021).

Triple-negative breast cancers or in short TNBCs are regarded as aggressive types of breast cancer and are the product of impaired expression of progesterone and estrogen receptors as well as human growth factor receptor 2 (Bianchini et al., 2016). According to the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines, TNBCs are typically characterized by cellular expression of progesterone and estrogen receptors of ≤1% and human growth factor receptor 2 expressions between 0 and 1+, as determined by immunohistochemistry (Wolff et al., 2014). There are four transcriptional subtypes of TNBCs: two basal subtypes, which are grouped as BL1 and BL2, a mesenchymal subtype M, and a luminal androgen receptor subtype

(Lehmann et al., 2016). Further, TNBC can be categorized into six different subgroups based on their molecular heterogeneity: immunomodulatory, luminal androgen receptor expression, mesenchymal stem-like, mesenchymal-like, basal-like, and unstable (Yam et al., 2017). TNBCs constitute 12–17% of all breast cancers and are naturally recurrent. Scientifically, this cancer is categorized as a distant subgroup within a broad category of breast cancers (Foulkes et al., 2010). The clinical behavior of TNBCs is relatively aggressive compared to that of other subtypes of breast cancer. Additionally, these cancers have characteristic metastatic patterns and poor prognosis (Dent et al., 2007). TNBCs represent 24% of newly diagnosed breast cancers, and a steady increase has been reported in their incidence (Tsai et al., 2016). It has been reported that in 2018 about 2,088,849 cases of TNBC were reported making it a common cancer in women (Singh et al., 2020). The average survival rate from the disease is ~10.2 months in perspective on the currently available therapy, with a 65% 5 years survival rate in cases of regional tumors and 11% for those where the tumor is spread to distant organs (Kohler et al., 2015).

In this review, the focus is to provide a comprehensive overview of TNBCs, epidemiology and risk factors, signaling pathways, prognosis, current and investigational treatment paradigm, and the role of artificial intelligence in TNBC diagnosis and treatment.

EPIDEMIOLOGY OF TNBC

TNBC accounts for 15–25% of all breast cancers (Yin et al., 2020). The TNBC proportion in all age groups followed a similar trend (Hudis and Gianni, 2011; Khan et al., 2017). However, younger and older women have increased rates of BRCA and basal TNBC and apocrine and neuroendocrine TNBC. African American and Hispanic women are found to be at high risk of TNBC, and African Americans have a worse prognosis compared to other groups (Reynolds, 2007). In a case study conducted in 2009, 187 TNBC patients were reported to have a 2.5% higher risk for TNBC who used oral contraceptives for more than 1 year. The risk is 4.2% among women aged less than 40 years. It was also noted that when the duration of oral contraceptive use increased, the risk increased. In the United States, TNBC is responsible for 12% of breast cancers, with a 5-years survival rate of 8–16% (Howard and Olopade, 2021).

POTENTIAL RISK FACTORS

The potential risk factors of TNBC can be divided into non-modifiable and modifiable risk factors.

NON-MODIFIABLE RISK FACTORS

Age

Approximately 80% of breast cancers (including TNBCs) are >50 years old (Donepudi et al., 2014). The cancer risk increases with age as follows: 1.5% risk at the age of 40 years, 3% at age 50, and more than 4% at age 70 years (Łukasiewicz et al., 2021). In addition, a relationship exists between cancer subtype and age. This can be explained by TNBC, which is mostly diagnosed in the age group of <40 years, whereas in patients aged >70 years, luminal A subtype cancer is more common (McGuire et al., 2015).

Sex

Due to different sex hormonal stimulation, female sex is considered a higher risk for TNBC compared to male sex. Females have breast cells that are very susceptible to estrogen and progesterone hormones, as well as imbalances. Circulation of estrogens and androgens is associated with an increased risk of breast cancer (Hormones et al., 2013). In the case of premenopausal and postmenopausal women, physiological changes in endogenous sex hormones result in a higher risk of breast cancer (Hormones and Group, 2002; Zhang et al., 2013). In men, the prevalence of breast cancer is 1%. The important factors which increase a man's risk of breast cancer are; older age, "BRCA2/BRCA1" mutations, and increased estrogen levels, genetic history in family, and highly exposure to radiation (Giordano, 2018).

Genetic Mutations

Mutations in genes such *as BRCA1* and *BRCA2* were found to be strongly associated with TNBC (Shiovitz and Korde, 2015). Mutations in *TP53*, *CDH1*, *PTEN*, and *STK11* are also associated with breast cancer and TNBC incidence (Corso et al., 2018; Shahbandi et al., 2020). Mutations in the *XRCC2* gene are also associated with high risk of breast cancer (Kluźniak et al., 2019). Further, it has been revealed that *BRCA1*-related tumors profile resembles the TNBC subtype, while the profile of the BRCA2-associated tumor correlates to luminal-like breast cancers, particularly the Luminal B subtype (Incorvaia et al., 2020).

Race/Ethnicity

The incidence of TNBC remains high among white non-Hispanic women (Hill et al., 2019). In addition, the mortality rate is significantly higher among black women, and black women are considered to have the lowest survival rates for malignancy (DeSantis et al., 2016).

Genetic History

Genetic history is one of the major risk factors associated with breast cancer (similar to TNBC). Approximately 13–19% of diagnosed breast cancer patients report a first-degree breast cancer relative (Cuzick, 2003). Moreover, the risk is higher in family members of age <50 years (Baglia et al., 2018). The genetic history of ovarian cancer in a family, particularly those with *BRCA1* and *BRCA2* mutations, has a greater risk (Wu et al., 2018).

Breast Tissue Density

As per clinical practice, breast tissue density has been categorized as low-density breasts, fatty, and high-density breasts. Women

receiving hormone replacement therapy are reported to have denser breasts during early age, during pregnancy, and breastfeeding, even with lower BMI (Titus-Ernstoff et al., 1998). In postmenopausal and premenopausal women, the density of the breast affects the risk of cancer, that is, the higher the density, the higher the chances (Checka et al., 2012). Breast tissue density screening could be a promising and quick approach for the rational surveillance (Kim et al., 2020).

History of Radiation Therapy

A history of radiotherapy can lead to the development of secondary tumors. This is mainly dependent on the patient's state and age (Ng and Shuryak, 2015). Patients aged <30 years are considered at higher risk (Zhang et al., 2020), and radiotherapy treatments, such as multiple-field IMRT (6F-IMRT) and double partial arc (VMAT) techniques can increase the chances of secondary tumors (Balaji et al., 2016). Radiotherapy in patients with a family history of breast cancer is considered to be at a higher risk (Bartelink et al., 2001).

History of Breast Diseases

The initial symptoms of cancer are cancerous lesions in the breast (Schacht et al., 2014; Muneer et al., 2019). Regarding the family history of disease, the other risk factors associated with breast cancer are; *in-situ* carcinoma, atypical hyperplasia, proliferative lesions and non-proliferative lesions (Dyrstad et al., 2015; Socolov et al., 2015). Breast cancer risks include a family history of breast cancer and benign lesions (Wang et al., 2004).

MODIFIABLE RISK FACTORS

Drugs

Diethylstilbestrol is a major cause of breast cancer during pregnancy (Hoover et al., 2011). Although much more study and research is required to support this statement, diethylstilbestrol intake and consumption by pregnant women not only causes breast cancer in the mother but also the child (Hilakivi-Clarke, 2014). This relationship is observed with diethylstilbestrol uptake even without the expression of estrogen and progesterone receptors (Palmer et al., 2006). The breast cancer risk increases with an increase in diethylstilbestrol doses. Female age is another consideration, that is, the risk increases 1.9 times in women older than 40 years (Narod, 2011). Hormonal replacement therapy, when carried out for more than 5–7 years, increases the chances of breast cancer. The continuous uptake of the selective antidepressants, paroxetine, tricyclic, and serotonin inhibitors, also increases the chances of breast cancer (Cotterchio et al., 2000). Similarly, Friedman reported tetracycline can increase risk of breast cancer (Friedman et al., 2006). Furthermore, the relationship between the risk of breast cancer and excessive use of hypertensive medications, anti-inflammatory non-steroidal drugs, and statins has also been studied, but the research data in this regard are not efficient in supporting these data (Coogan et al., 1999; Denoyelle et al., 2001).

Body Mass Index

According to several epidemiological studies, obesity is a potential risk factor for breast cancer. Epidemiologically, estrogen receptor-positive breast cancer develops in obese women in the postmenopausal period (Kolb and Zhang, 2020). However, women more than 50 years of age with greater BMI are at higher risk of breast cancer than those with low BMI (Wang et al., 2019). However, it has been reported that people with a higher BMI are at a high risk of tumors with a high percentage and size of lymph node metastasis. In premenopausal women, obesity is not only an evident cause of cancer, but also high mortality (James et al., 2015). Procarcinogenic events are facilitated by greater fat content in the body, which in turn enhances the circulation of hormones and inflammation. Females with a BMI greater than 25 kg/m^2 had poor clinical outcomes (Protani et al., 2010). Greater fat contents, although with the relevant BMI in post-menopausal women, have poor clinical outcomes. People with a family history of breast cancer are at a greater risk of breast cancer with greater BMI (Iyengar et al., 2019).

Physical Activity

The physical activity is considered the best action to be performed in order to prevent breast cancer (Kyu et al., 2016). This is supported by the study of Chen et al. that in women the breast cancer occurrence is reduced by physical activity during the postmenopausal period (Hormones et al., 2013), The Physical activity reduces the exposure to endogenous sex hormones and can also alter insulin-like growth factor-1 levels and immune responses (Hoffman-Goetz et al., 1998; Hormones et al., 2013).

Alcohol Intake

Various studies reported alcohol consumption is a major cause of cancer in the gastrointestinal tract, along with breast cancer (Rachdaoui and Sarkar, 2013). Alcohol and alcohol beverages can increase the risk of malignancy. The hormone balance is disturbed along with the enhanced production of estrogen, which in turn increases body weight. Alcohol and its beverages are considered to increase the risk of cancer growth (Coronado et al., 2011). Alcohols are the major causative agents of estrogen-positive breast cancers (Zeinomar et al., 2019). Morphological alterations of the breast and its tissues have been reported with the consumption of alcohol before the 1st pregnancy (Liu et al., 2015).

Insufficient Vitamin Supplements

Vitamins are anti-cancer elements that can prevent breast malignancies. Research is underway to evaluate the risk of cancer with the consumption of vitamins, particularly vitamin B, C, and E folic acids and multivitamins (Cui and Rohan, 2006). Vitamin D supplements, that is, high serum 25-hydroxyvitamin D, are thought to be potential cancer control agents in postmenopausal women and in the premenopausal period (Atoum and Alzoughool, 2017). Excessive expression of vitamin D receptors is associated with a lower mortality rate in patients with breast cancer (Zhou et al., 2020). Artificial light exposure for a longer duration can increase the risk of breast

malignancy (Al-Naggar and Anil, 2016). This occurs because of the activation of melatonin pigments and consequent epigenetic shifts (Johns et al., 2018).

Exposure to Chemicals and Drugs

Females who have been exposed to dreadful carcinogenic chemicals are at higher risk of breast cancer and epigenetic alterations and mutations. Exposure and duration of exposure contribute to an increased risk of breast cancer mutagenesis (Casey et al., 2015). Exposure of mammary glands to polychlorinated biphenyl (PCB) and dichlorodiphenyltrichloroethane (DDT) chemicals increases the risk of breast cancer (Leso et al., 2019). Furthermore, continuous exposure to organic solvents, insecticides, and oil mist increases the risk of breast cancer (Videnros et al., 2020). Antibiotics, statins, antidepressants, and antihypertensive drugs can increase the risk of breast cancer. Similarly, NSAIDs that contain aspirin and ibuprofen are considered major risk factors for breast cancer (Brandes et al., 1992; Bjarnadottir et al., 2013).

Smoking

Tobacco causes mutations in oncogenes and p53 suppressor genes (Terry and Rohan, 2002). Active smoking passive smokers are at a risk of cancer. Smoking during pregnancy and chain smokers are at potential risk of malignancies (Couch et al., 2001).

Intake of Processed Food/Diet

According to the WHO, processed foods, such as meat, are confirmed group-1 carcinogen for gastrointestinal cancer and breast malignancy (Dandamudi et al., 2018). The excessive use of saturated fats is also considered a carcinogen. The obesity-causing ultra-processed diet plans that are enriched in elements such as sugar, sodium, and fats are thought to be carcinogenic and increase the risk by 11% (Fiolet et al., 2018). Diets that are rich in green vegetables, fresh fruits, protein-enriched grains, and legumes are anti-carcinogenic and therefore reduce the risk of breast cancer (Castello et al., 2014). Similarly, diets rich in phyto-estrogen, folate elements, saturated fibers, n-3 PUFA, and vitamin D are regarded as anti-cancer agents (Dunneram et al., 2019). Hence, a low dose consumption of saturated fat and n-6 PUFA has been proposed (Li et al., 2014). The antioxidants found in green tea have also shown anti-carcinogenic properties (Liu and Chen, 2013). Curcuminoids and sulforaphane (SFN) derived from turmeric are thought to be anti-carcinogens (E Wright et al., 2013).

Complexities of TNBC Metastasis

TNBC is one of the most aggressive subtypes of cancer that is often associated with poor patient outcomes because of the development of metastases in secondary organisms like in the brain, bone, and lungs (Zeichner et al., 2016). Metastatic growth to these distant organs, represents a significant clinical challenge, as metastatic disease is currently incurable and is a primary death cause for the vast majority of TNBC patients. Metastatic spread of cancer is a complex, poorly understood process, and involves multiple steps, such as angiogenesis acquisition of invasive properties through epigenetic and genetic alterations, intravasation through the basement membrane, extravasation of some cancer cells to distal tissues, and tumor-stroma interactions (Nguyen and Massagué, 2007; Fatima et al., 2018). Metastatic cells outgrowth in a foreign tissue environment is considered as the rate-limiting step of breast cancer metastasis and in this stage, breast cancer cells are difficult to detect and show resistance to chemotherapy due to lack of proliferation (Giancotti, 2013; Dujon et al., 2021). To date, this remains a clinical obstacle, since the patients considered as "survivors" can develop metastatic tumors years later. Disseminated tumor cells can enter a state of dormancy in the secondary organs by achieving a balanced state of proliferation and apoptosis. Successful emergence from dormancy is the result of further evolution of surviving disseminated tumor cells by accumulating molecular changes as well as *via* permissive interactions with the tumor microenvironment (Giancotti, 2013). By achieving these characteristics, metastatic tumor cells can optimally adapt to the host microenvironment and initiate colonization. While significant information has been generated to identify the specific processes required for breast cancer initiation, still, much effort to elucidate the molecular mechanisms and roles of critical genes and signaling pathways involved in the late stages of metastatic growth are required.

SIGNALING PATHWAYS

Notch Signaling Pathway

The term Notch was first described by Thomas Hunt Morgan in 1917 and refers to transmembrane receptors and ligands (Gu et al., 2016; Fatima et al., 2018). This juxtacrine signaling pathway plays a central role in the developmental process and uses communication among cells *via* transmembrane interactions with ligands (Yaron et al., 2014). The Notch pathway is a short-range cell-to-cell communication pathway that is critical for metazoan development (Artavanis-Tsakonas et al., 1999). The Notch receptor is expressed on the plasma membrane and cleaved by furin-like convertase in the Golgi compartment (Blaumueller et al., 1997). The Notch pathway has been identified in *Drosophila melanogaster*. From a structural perspective, Notch receptors share three domains: an intracellular domain, a transmembrane region, and an extracellular domain (Bellavia et al., 2008). This signaling pathway is key in cell proliferation and differentiation (Palomero et al., 2006) most importantly, governs embryonic development and maintains tumor stemness to TNBC tumor metastasis. There are four Notch receptors and five ligands in this pathway. The receptors can be named as Notch 1 to 4, while ligands are Delta-like 1, Delta-like 3, Delta-like 4, Jagged-1, and Jagged-2 (Speiser et al., 2013). Several reports have indicated the overexpression of Jagged 1 and Delta 1 in breast cancer. The Notch 1 is involved in pancreatic cancer and hematological malignancies, while Notch-3/4 has been found to assist in tumor proliferation and survival. Notch-2 overexpression in TNBC appears to play a protective role (Weijzen et al., 2002). Notch expression has been linked to TNBCs, and scientists believe that targeting receptors by monoclonal antibodies can

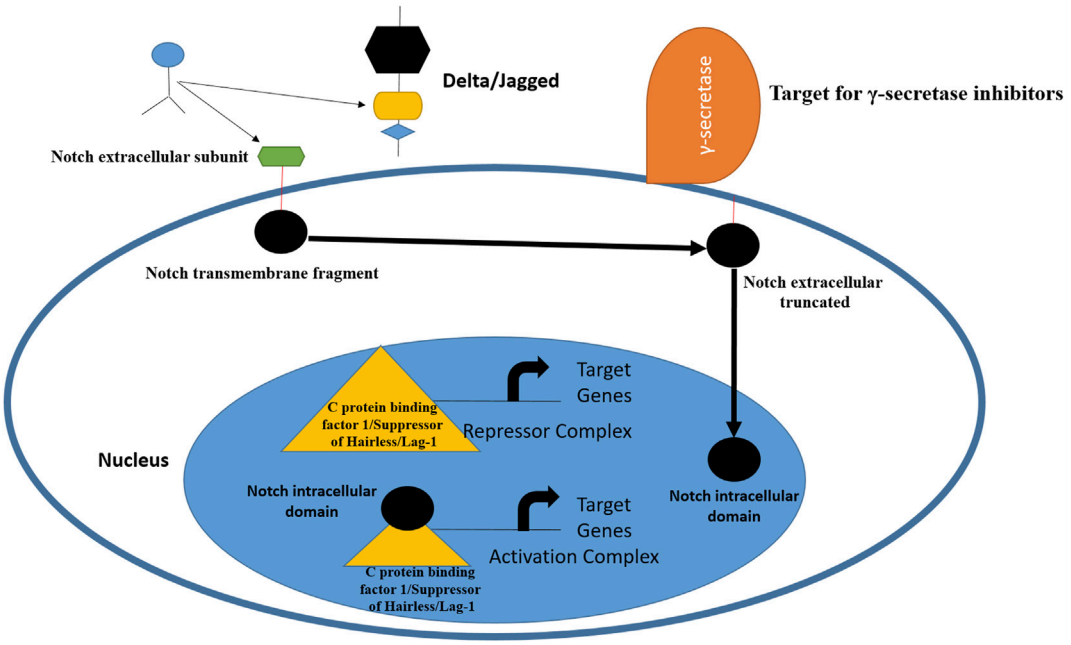

FIGURE 1 | Schematic representation of Notch receptor activation and avenues for therapeutic intervention. The pathway is activated by ligand binding to Notch receptor, followed by proteolytic cleavage fy proteases. This releases the Notch intracellular domain, which is then transferred to nucleus for biding with C protein binding factor 1/Suppressor of Hairless/Lag-1 allowing conversion of complex from repressor to activator of Notch genes. From inhibition perspective, γ-secretase inhibitors and monoclonal antibodies can inhibit Notch ligands and receptors (Yuan et al., 2015; Medina et al., 2020).

reduce HES and HEY-L families (Sharma et al., 2012). The monoclonal antibody based on the delta-like ligand 4 Notch ligand also showed effectiveness in treating TNBC (Benedito et al., 2009). Inhibitors that target the Notch signaling pathway act at proteolytic cleavage and block the formation of multimeric γ-secretase complexes; thus, these drugs are termed γ-secretase inhibitors (Chan et al., 2007). The Notch pathway and the points at which it can be blocked are shown in **Figure 1**.

Wnt/β-Catenin Pathway

Different Wnt ligands, such as WNT3A, WNT11, and WNT5A, are reported to be pertinent in cancer migration and invasion (Zhu et al., 2012). In particular, the FZD6 receptor is associated with increased malignant cell motility in TNBC (Corda et al., 2017). It has been revealed that OMP-18R5 antibody targets Frizzled receptors to diminish tumor cell proliferation in colon, lung, breast, and pancreatic tumors (Gurney et al., 2012). Wnt/β-catenin signaling pathway is activated in epithelial ovarian cancer and targets gene regulate cell proliferation and apoptosis thereby mediating cancer initiation and progression. Furthermore, Wnt inhibitors can destroy drug-resistant cells and cancer stem cells (Dean et al., 2005).

TGF-β Signaling Pathway

TGF-beta 1 is expressed exponentially in TGF-β1 and TGF-β1 and has been implicated in its important role in breast cancer stem cells (Jamdade et al., 2015). *In vivo* analysis, inhibition of TGF-β leads results in multiplication and growth of tumor cells. The frequent overexpression of TGF-β in the TNBC tumor microenvironment, particularly in stromal, tumor-associated immune cells, and tumor cells. In these cells, SMAD4 and SMAD2/3 cause metastasis and angiogenesis (Bhola et al., 2013). Thus, inhibition of TGF-β plays a significant therapeutic role in patients with metastasis.

Signaling Pathway of CSPG4 Protein

The CSPG4 protein (non-glial antigen) is expressed as a cell surface proteoglycan by basal breast carcinoma cells. Therapeutically, CSPG4 inhibition allows for efficient management of breast cancer (Wang et al., 2010). Monoclonal antibodies can block the CSPG4 protein, which hinders survival signaling pathways in tumor cells. In addition, controlling the overexpression of CSPG4 by targeting it therapeutically is seen in different TNBC cells (Cooney et al., 2011).

Hedgehog Signaling Pathway

The Hedgehog signaling pathway is involved in cancer cell invasion, metastasis, drug resistance, and tumor recurrence (Li et al., 2012). Overexpression of this pathway results in poor prediction of breast cancer mortality, especially in TNBC patients. The Hedgehog signaling pathway is considered to initiate breast cancer malignancy. Thiostrepton is a novel experimental drug that suppresses TNBC $CD44^{+}/CD24^{-}$ cancer stem cells (Yang et al., 2016).

PI3K/AKT/mTOR Pathway

Rapamycin and paclitaxel drugs are used to inhibit the PI3K/AKT/mTOR pathway and hence play a significant role in TNBC

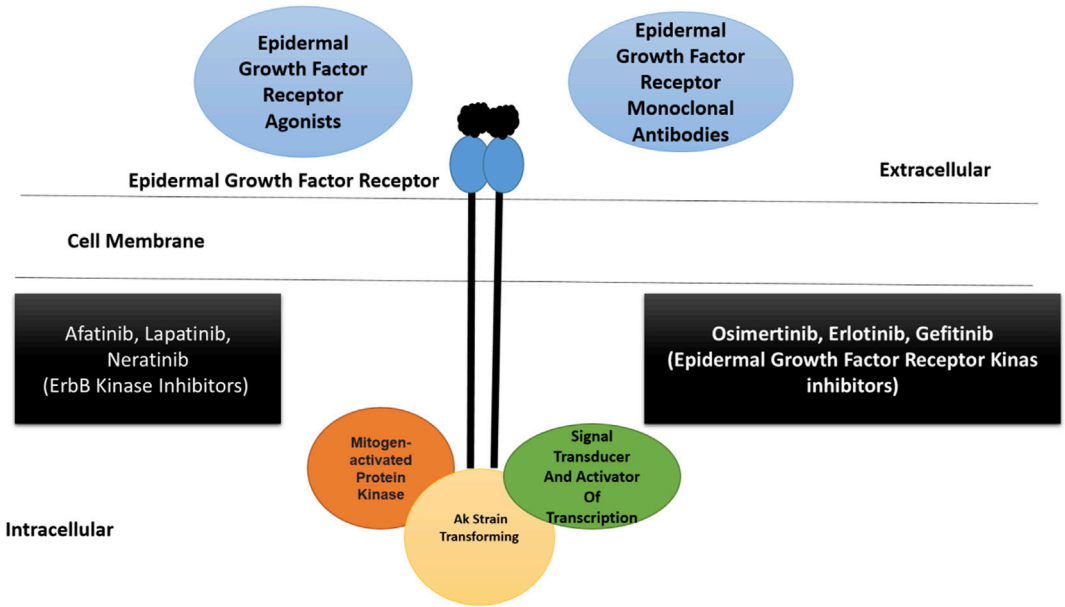

FIGURE 2 | A schematic illustration of epidermal growth factor receptor signaling pathway along with activators and inhibitors. The pathway is part of ErbB superfamily. The epidermal growth factor receptor is able to bind to different ligands at the extracellular surface resulting in the activation of downstream signaling events. Therapeutics including monoclonal antibodies and different kinase inhibitors can block the binding of ligand to the receptor. The kinase inhibitors can also block the function of other ErbB receptors (Ali and Wendt, 2017; Medina et al., 2020). Inhibitors of polyadenosine diphosphate-ribose polymerase.

treatment. Furthermore, mTOR antibodies are considered more effective than mTOR inhibitors alone (Ali and Wendt, 2017). In TNBC patients, ipatasertip (an AKT inhibitor) can promote progression-free survival by inactivating the PI3K/AKT pathway. Despite these efforts on PI3K/AKT/mTOR pathway inhibitors, synthesis of novel inhibitors is needed to block the PI3K/Akt/mTOR pathway and act as therapeutic agents against TNBC (Blanco et al., 2014).

Epidermal Growth Factor Receptor

The epidermal growth factor receptor is reported in 89% of TNBC and is considered an attractive therapeutic target, particularly in BL2 subtype tumors (Sobande et al., 2015). The expression of this gene results in primary tumorigenesis and metastasis. The EGFR inhibitor gefitinib lowers the proliferation of cancer cells and increases carboplatin and docetaxel cytotoxicity (Eccles, 2011). Several EGFR inhibitors, such as lapatinib and erlotinib, are currently being tested against TNBC, in addition to cetuximab and panitumumab (monoclonal antibodies) (Nabholtz et al., 2014; Hsiao et al., 2015). The synergistic therapeutic approach of monoclonal antibodies and chemotherapeutics is considered to be more effective. This can be exemplified by the combined use of carboplatin and cetuximab, and cisplatin and cetuximab proved to be more efficacious in patients with advanced TNBC (Carey et al., 2012). Additionally, tri-inhibitor therapy, including carboplatin, gefitinib, and docetaxel, enhances TNBC cytotoxicity. Cannabidiol inhibits breast cancer metastasis by interfering with the epidermal growth factor pathway (Velasco et al., 2016). The epidermal growth factor receptor signaling pathway is presented along with activator and inhibitor points of action, as shown in **Figure 2**.

Polyadenosine diphosphate-ribose polymerase is involved in molecular mechanisms that allow cells to recover from DNA damage, apoptosis, gene transcription, and genomic stability (Park and Chen, 2012). In approximately 70 and 23% of breast cancers, BRCA1 and BRCA2 mutations have been reported (Mahfoudh et al., 2019). For both these mutations and TNBC, PARP inhibitors are regarded as the most vital drugs. The activation of PARP-1 and PARP-2 proteins is a consequence of DNA strand breaks. The polyadenosine diphosphate-ribose polymerase enzyme is synthesized by PARP and drives the single-strand break repair and excision repair pathway (De Vos et al., 2012). It has been observed that when PARP activity is affected, it blocks DNA polymerase ε for DNA damage repair (Helleday, 2011). Veliparib and olaparib are both PARP inhibitors that have different catalytic inhibition mechanisms (Murai et al., 2012). The inhibition of poly (ADP-ribose) polymerase in BRCA-1/2-associated and sporadic cancers is shown in **Figure 3**.

Mammalian Target of Rapamycin

The mTor pathway is responsible for poor prognosis due to the aggressive nature of the cancer and its good tissue invasion property (Xie et al., 2016). Errors in the mTOR pathway are strongly correlated with malignancy (Fruman and Rommel,

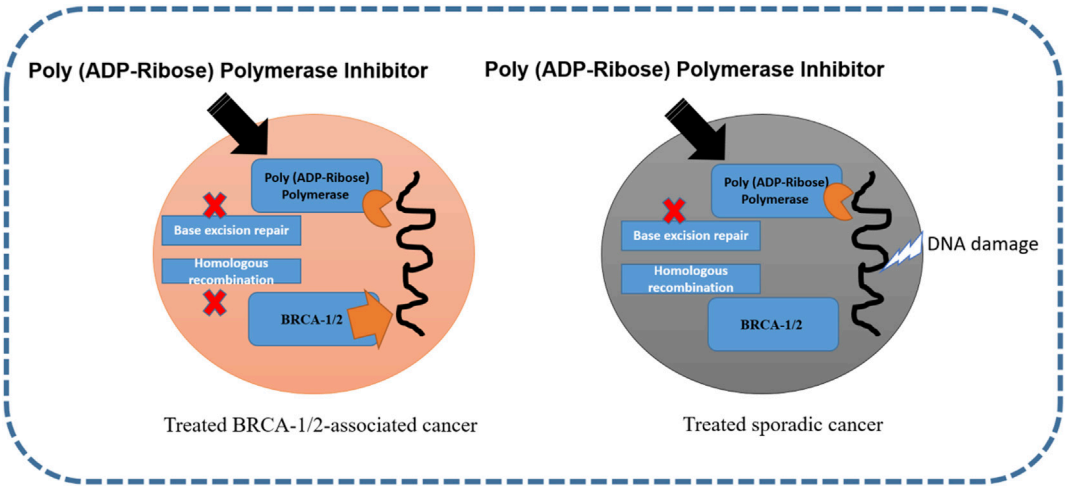

FIGURE 3 | Inhibition of poly (ADP-ribose) polymerase in BRCA-1/2-associated and sporadic cancers (Ellisen, 2011; Medina et al., 2020).

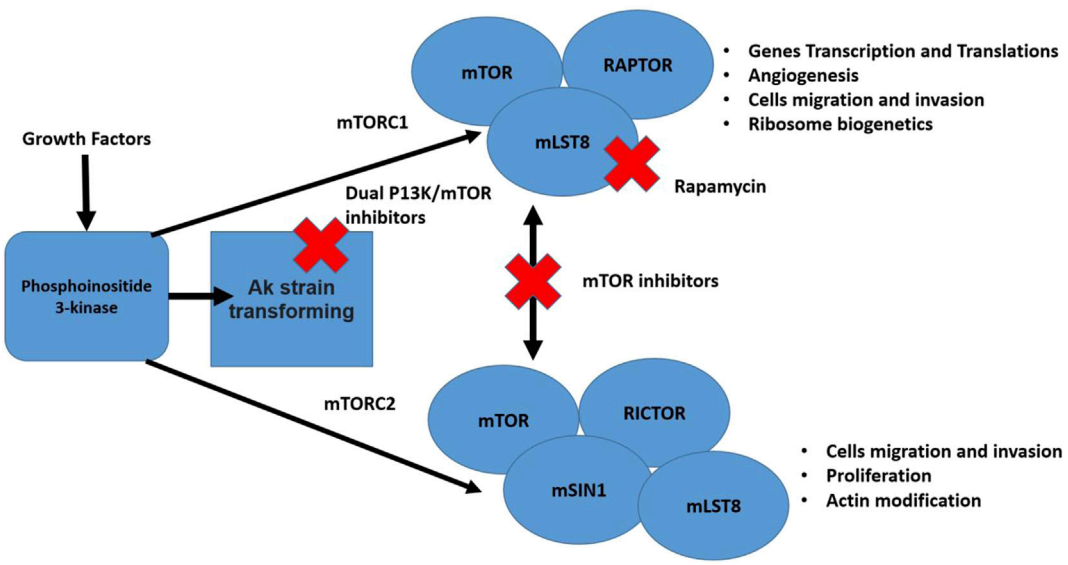

FIGURE 4 | The mTor pathway illustrating two distinct complexes (mTORC1 and mTORC2). The pathway is stimulated by different growth factors. The mTORC2 activated Akt. Multiple cell functions are regulated by both mentioned complexes that are considered vital for cancer development. Also, in the figure different steps of the pathway that can be blocked by inhibitors are shown (Xie et al., 2016; Medina et al., 2020).

2014). The phosphorylation reactions of this pathway are also associated with proliferation, vascular endothelial growth factor, and angiogenesis, that enhance endothelial cell growth (Arcaro and Guerreiro, 2007). Moreover, high expression of a protein kinase enzyme (Akt) has been reported to be involved in tumor invasion and metastasis therefore, inhibiting the mTOR pathway can be an efficient anti-cancer strategy for several human malignancies (Xie et al., 2016). In general, inhibitors of the PI3K/AKT/mTOR network can be grouped as: 1) AKT blockers, 2) Pan-PI3K/mTOR blocker, 3) PI3K blocker, 4) Rapalogs (temsirolimus, everolimus, and deforolimus), and 5) mTOR blocker (Xie et al., 2016). The mTor pathway and checkpoints where it can be blocked are presented in **Figure 4**.

Immunosuppressive Immune Cells in the TNBC Tumor Microenvironment

The tumor microenvironment (TME) involves the surrounding blood vessels, fibroblasts, immune cells, signaling molecules and the extracellular matrix around the tumor (Deepak et al., 2020). Tumor Infiltrating Lymphocytes (TILs) produce endogenous antitumor immune response for inhibiting tumor progression

TABLE 1 | Conventional treatment option for TNBC.

Treatment	TNBC Type	Drugs	References
Neo-adjuvant Therapy	Early TNBC, Advanced or Metastatic	Capecitabine + Taxane Anthracyclines + Taxanes Capecitabine + Ixabepilone /Ixabepilone monotherapy	Ali and Wendt, (2017)
New Neo-adjuvant agents	BRCA mutations	Nab-paclitaxel, evacizumab, Carboplatin	Sikov et al. (2015)
Adjuvant agents	Early TNBC	Taxanes and Anthracyclines	Martin et al. (2013)

and improving free survival rate of TNBC patients (Reis-Filho and Tutt, 2008; Yu and Di, 2017). Tumor associated macrophages are important for immunosuppressive role by secreting inhibitory cytokines, regulatory T cells infiltration promotion, and reactive oxygen species reduction (Yu and Di, 2017). Cancer-Associated Fibroblasts lower anti-tumor immunity, favor tumor cell proliferation and invasion and reshape the extracellular matrix (Yu and Di, 2017). Tumor associated neutrophils aid in lysing tumor cells and induce antitumor function (Annaratone et al., 2020).

Existing and Investigational Treatment Paradigm

From a chemotherapeutic perspective, TNBC is very sensitive, and treatments require extreme care. Common treatment involves the use of alkylating agents (such as cyclophosamide), anthracycline (doxorubicin topoisomerase blocker and DNA intercalating agents), anti-metabolite fluorouracil, and anti-microtubule agent (taxane) (Chang et al., 2019; Won and Spruck, 2020). For early diagnosis of TNBC, neoadjuvant chemotherapy and subsequent surgery are applied. No standard chemotherapy has been described for the treatment of relapsed TNBC. Treating advanced TNBC includes the following drugs: gemcitabine and capecitabine (anti-metabolites), eribulin (non-taxane microtubule inhibitor), and platinum (DNA cross-linker). The conventional treatment options for TNBC are listed in **Table 1**.

For advanced TNBC, new therapies have been reported, particularly when surgery is not desired. Compared to other breast cancer subtypes, TNBCs show greater immunogenicity. They have tumor-infiltrating lymphocytes in their microenvironment and express programmed cell death ligand (PD-L1) in high order (Stanton et al., 2016). Considering the therapeutic potential of the PD-L1 pathway, several immunotherapies have been explored, and atezolizumab combined with nanoparticle albumin-bound paclitaxel has been approved by the US FDA as the first-line therapy in 2019 (Schmid et al., 2018). Pembrolizumab was approved in 2017 as an anti-PD-1 antibody (Dudley et al., 2016). In 2018, talazoparib and olaparib were approved by the FDA for treating HER2 negative breast cancer (Won and Spruck, 2020). With the aim of improving TNBC treatment, several therapeutic strategies have been explored in clinical studies, including those that target or are immune specific for tumor stroma, intracellular or surface receptors, DNA damage response, and signaling pathways. So far, 399 studies have been shown on ClinicalTrials. gov for TNBC and are under phase III investigation (Ahmed et al., 2014; Riaz et al., 2017; Won and Spruck, 2020). The concept of immune checkpoint inhibitors is to halt regulatory immune checkpoints and thus activate anti-tumor responses. This treatment strategy is considered a game changer in cancer therapy and involves molecules that can negatively alter the immune response. Immune checkpoints can be readily blocked by antibodies or modified by recombinant ligands (Pardoll, 2012; Muneer et al., 2021). Immune checkpoint inhibitory antibodies aid in the attachment of molecules capable of stopping the immune response to tumor-infiltrating lymphocytes, leading to reactivation of antitumor immune responses (Singh et al., 2021). Research regarding the use of immune checkpoint inhibitor(s) either alone as a single agent or in combination therapy is ongoing (Adams et al., 2019). Neoadjuvant therapy has yielded mixed results. Promising anti-tumor activity and considerable safety were noted when neoadjuvant chemotherapy was used in combination with pembrolizumab in early stage TNBC (Nanda et al., 2020). In one study, neoadjuvant combination therapy revealed a higher pathologic complete response rate (65%) than the placebo-chemotherapy group (61%). In another study, neoadjuvant chemotherapy was investigated with or without atezolizumab for early stage and high-risk unilateral breast cancer. However, no improvement was seen in the pathologic complete response using the combination therapy (Gianni et al., 2019). Immunotherapy involving targeting of the 2B receptor ($A_{2b}R$) and adenosine 2A receptor ($A_{2a}R$) is considered a promising approach for the reactivation of antitumor immunity and enhancement of cytotoxic T cell immune responses (Ohta, 2016). In different clinical trials, the combination of immune checkpoint inhibitors and adenosine pathway inhibitors has been investigated. For example, NZV930, which is an anti-CD72 antibody when used alone or in combination with a PD-1 inhibitor or $A_{2a}R$ antagonist to block adenosine mediated inhibition of T lymphocytes. In another ongoing study, NIR178 was used in combination with spartalizumab for diffuse large B-cell lymphoma and multiple solid tumors to check whether the addition of an antagonist improves PD-1 inhibition efficacy. In addition, AB928 (a dual adenosine $A_{2a}R/A_{2b}R$ receptor antagonist) is being evaluated in combination with AB122 (PD-1 inhibitor) for patients with advanced malignancies (Powderly et al., 2019). Different types of poly (ADP-ribose) polymerase inhibitors have also been described. These inhibitors include niraparib, veliparib, olaparib, talazoparib, and rucaparib (Won and Spruck, 2020).

TABLE 2 | Different types of nano medicines under experimental and clinical testing for TNBC theranostics (Medina et al., 2020).

Nanoparticle	Status	Therapeutic applications	References
Fluorescent nano-diamonds	Experimental and under clinical testing	It uses non-radioactive materials for imaging and has enhanced specificity and sensitivity	Fudala et al. (2013)
Quantum dots	Experimental and under clinical testing	Quantitative detection and cancer imaging	(Michalet et al., 2005; Zheng et al., 2016)
Silver nanoparticles	Experimental and under clinical testing	Cytotoxicity to cancer cells	Swanner et al. (2015)
Superparamagnetic iron oxide nanoparticles	Experimental and under clinical testing	Induce tumor apoptosis	Vyas et al. (2015)
Iron oxide nanoparticles	Experimental and under clinical testing	Produce contrasting images in MRI	Hayashi et al. (2013)
Gold nano-stars	Experimental and under clinical testing	Drug delivery, hyperthermia, theranosics and gene therapy	Zhang et al. (2021)
Core-shell nanoparticles	Experimental and under clinical testing	Generate apoptosis	Meng et al. (2018)
Nanocages	Experimental and under clinical testing	Hyperthermia, Immunotherapy, theranostics, and photodynamics	Liang et al. (2018)
Nanocomposites	Experimental and under clinical testing	Hyperthermia, Immunotherapy, theranostics, drug delivery and photodynamics	Liao et al. (2019)
Nanorods	Experimental and under clinical testing	Hyperthermia, Immunotherapy, gene therapy, theranostics, drug delivery and photodynamics	Feng et al. (2015)

The role of androgen receptor inhibitors in TNBC still needs to be explored, and more insights need to be explored. The first-generation androgen receptor antagonist bicalutamide is a proof of concept for treating advanced TNBC, and the results showed a modest clinical benefit rate of 19% (Gucalp et al., 2013). Abiraterone, a second-generation anti-androgen inhibitor, shows promising targeting of androgen biosynthesis (Bonnefoi et al., 2016). Another second-generation androgen receptor inhibitor, enzalutamide, showed competitive binding to the androgen receptor ligand-binding domain and blocked its nuclear translocation, coactivator recruitment, and DNA binding (Traina et al., 2018). Seviteronel targets estrogen and androgen production (Gucalp et al., 2017). In addition to these therapeutic options, cell surface targets, such as tumor-associated carbohydrate antigens, have been explored as antigens for vaccine formulation. In particular, the Globo H antigen, which is expressed on the surface of different cancer types, can be explored for vaccine design. Adagloxad simolenin is an immunostimulating agent consisting of a Globo H hexasaccharide epitope fused with a keyhole limpet hemocyanin protein carrier (Gilewski et al., 2001). The antibody-drug conjugate remains stable in plasma, attacking antigens at the tumor cell surface with high specificity and affinity, followed by internalization, cleavage, and release of the payload drug to exert anti-tumor activity. For example, Sacituzumab govitecan-hziy targets human trophoblast cell-surface antigen 2, which is present in more than 90% of TNBCs. In this case, the active metabolite is irinotecan in conjugation with anti-trophoblast cell-surface antigen 2 antibody (Bardia et al., 2019). Ladiratuzumab vedotin or a short LV main target is a transmembrane protein (LIV-1). The protein has metalloproteinase and zinc transporter activity and is expressed in more than 90% of breast tumors. Ladiratuzumab vedotin comprises the microtubule-disrupting agent monomethyl auristatin E as payload (Bonnefoi et al., 2016). In addition to these therapeutic strategies against TNBCs, new platforms have been described. In this approach, EZH2 inhibitors were evaluated against the CDK2-EZH2 axis, thus reactivating ERα expression (Nie et al., 2019). In a recent study, researches combined the PARP inhibitor with CSF-1R blocking antibodies to elucidate the key contribution of immunosuppression to limiting the effective anti-tumor response their study demonstrates that combining the PARP inhibition with macrophage targeting therapy induces a durable reprograming of the tumor microenvironment and can be used as a promising therapeutic strategy for TNBC (Mehta et al., 2021). In another new technique, the combination of histone deacetylase and DNA methyltransferase inhibitor results in ERα expression in breast cancer models (Yang et al., 2001). The different types of nanomedicines under experimental and clinical testing for TNBC theranostics are tabulated in **Table 2**.

Targeting Tumor Microenvironment for TNBC Therapy

The development of TNBC has strong association with the physiological state of TME. TNBC has been characterized with unique TME and is different from other subtypes (Roma-Rodrigues et al., 2019). TME has strong association with induction of angiogenesis, proliferation, apoptosis inhibition, suppression of immune system and resistance to drugs (Kuroda et al., 2021). The exosomes function as promising nanovesicles that directs TME orchestration by communicating cells within TME milieu (Deepak et al., 2020).

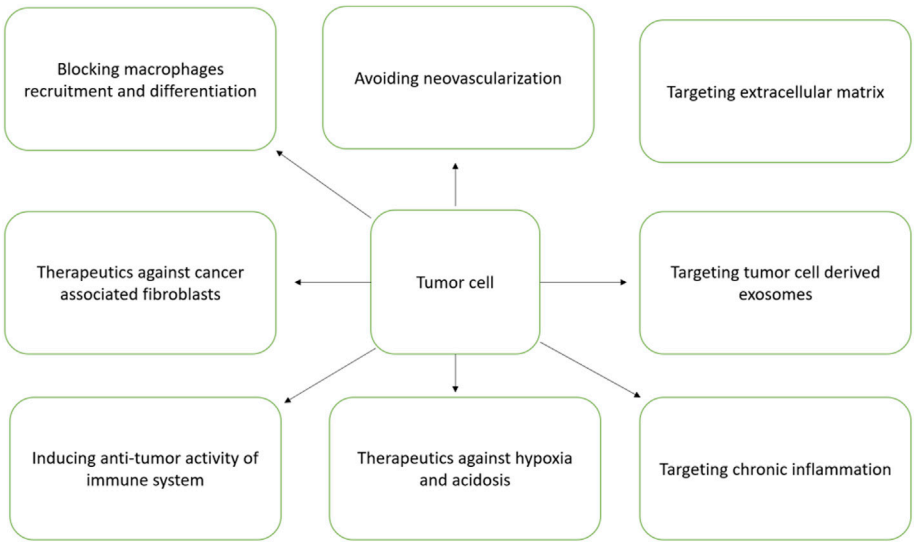

FIGURE 5 | Different TME targets for therapeutic intervention.

The different components of TME particularly the soluble factors, transformed extracellular matrix, immune suppressive cells, re-programmed fibroblasts and epigenetic modifications altogether helps in TNBC progression and metastasis (Deepak et al., 2020). Hence, TME is regarded as a good therapeutic target. The different TME targets for therapeutic intervention is schematically presented in **Figure 5**.

Prognosis of TNBC

The prognostic research of TBNC is mostly performed as retrospective studies, and these studies considered diagnostic data. Most of these studies used the triple negativity inclusion factor and neglected molecular markers for basal-like breast cancer. Poor prognosis has been observed in patients with TNBC (Nofech-Mozes et al., 2009). In contrast to other subtypes, TNBC development occurs more frequently in pre-menopausal women during early life. TNBC has a more aggressive expression profile (high p54 and Ki67 and low Bcl-2 expression), large tumor size, and high nuclear mitotic grade (Rhee et al., 2008). Many studies have demonstrated lower RFS in TNBC than in non-TNBC patients. The 4-year survival rate of TNBC patients was 85.5%, which is comparable to that of non-TNBC patients (94.2%) (Rhee et al., 2008). In another study, relapse frequency was less frequently reported in TNBC (Parikh et al., 2008). Tumor recurrence is 1.2 years which is shorter than that in non-TNBC patients. Similarly, TNBC has a worse prognosis in patients with recurrent breast cancers. The risk of tumor recurrence and death is high in TNBC, in contrast to the other types. It has been reported that the hazard ratio (HR 4.2 for developing TNBC tumor recurrence when compared to other cancers (Mersin et al., 2008). For triple-positive breast cancer, the 5 year survival is 91%, whereas for TNBC and HR-positive/HER2-negative cancers, it is 81 and 94%, respectively, (Kaplan and Malmgren, 2008).

Artificial Intelligence in TNBC Management

In the last decade, the power of artificial intelligence in different scientific fields, particularly in medicine, has been seen as a real tool for the efficient diagnosis and management of diseases. Artificial intelligence-based applications in medical sciences include computer-aided detection and diagnosis of diseases, case-based reasoning, explainable artificial intelligence, osteodetect machine learning, and rainbox boxes (Wang et al., 2018). Decision Support and Information Management System for Breast Cancer (DESIREE) is a product of artificial intelligence that can be used in cancer prediction and interpretation of clinical data. Different artificial intelligence-based tools are also used for breast cancer screening. The metadata, which serve as the basis of these artificially based algorithm designs, are provided by the cancer data bank along with patient biodata history and treatment trends. The data are analyzed both quantitatively and qualitatively to train the algorithms so that the tools/servers can assist physicians in faster and more accurate ways to detect and manage complex cancer types. Recently, Fernández Martínez et al. reported a machine-learning algorithm that is capable of detecting different TNBC subtypes. The Cancer Genome Atlas (TCGA) is another platform that collects SNP data, DNA and RNA sequence data, and reserve phase protein array information. Together with the Molecular Taxonomy of the Breast Cancer International Consortium (METABRIC), TCGA aims to provide data related to molecular heterogeneity of different breast cancer subtypes. Both databases could help oncologists in advising personalized medicine for cancer patients in a timely manner. Very limited work has been done so far in this field, and much more interdisciplinary efforts are required to put solid platforms that cannot easily diagnose TNBC early, but at the same time allow efficient and effective treatment.

CONCLUSION

Among all breast cancers, TNBC has the worst prognosis, in addition to suppressed immunity of the patient. Hence, understanding the TNBC molecular signaling pathway in the tumor microenvironment by applying multidisciplinary research will greatly advance TNBC diagnosis and therapy. In-depth information of TNBC pathways at the genetic and proteomic levels can assist in novel therapeutic design and successful trial development. New developments in computer biology and discoveries in immunology, nanotechnology, and molecular biology will ease earlier diagnosis and personalized treatment.

AUTHOR CONTRIBUTIONS

NA planned, performed and write whole review.

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frontiers in Molecular Biosciences

ORIGINAL RESEARCH
published: 14 February 2022
doi: 10.3389/fmolb.2022.783735

The Prognostic Value of the Developmental Gene FZD6 in Young Saudi Breast Cancer Patients: A Biomarkers Discovery and Cancer Inducers OncoScreen Approach

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Edited by:
Liaqat Ali,
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Specialty section:
This article was submitted to Molecular Diagnostics and Therapeutics, a section of the journal Frontiers in Molecular Biosciences

Received: 26 September 2021
Accepted: 05 January 2022
Published: 14 February 2022

Citation:
Assidi M, Buhmeida A, Al-Zahrani MH, Al-Maghrabi J, Rasool M, Naseer MI, Alkhatabi H, Alrefaei AF, Zari A, Elkhatib R, Abuzenadah A, Pushparaj PN and Abu-Elmagd M (2022) The Prognostic Value of the Developmental Gene FZD6 in Young Saudi Breast Cancer Patients: A Biomarkers Discovery and Cancer Inducers OncoScreen Approach. Front. Mol. Biosci. 9:783735. doi: 10.3389/fmolb.2022.783735

Wnt signalling receptors, Frizzleds (FZDs), play a pivotal role in many cellular events during embryonic development and cancer. Female breast cancer (BC) is currently the worldwide leading incident cancer type that cause 1 in 6 cancer-related death. FZD receptors expression in cancer was shown to be associated with tumour development and patient outcomes including recurrence and survival. FZD6 received little attention for its role in BC and hence we analysed its expression pattern in a Saudi BC cohort to assess its prognostic potential and unravel the impacted signalling pathway. Paraffin blocks from approximately 405 randomly selected BC patients aged between 25 and 70 years old were processed for tissue microarray using an automated tissue arrayer and then subjected to FZD6 immunohistochemistry staining using the Ventana platform. Besides, Ingenuity Pathway Analysis (IPA) knowledgebase was used to decipher the upstream and downstream regulators of FZD6 in BC. TargetScan and miRabel target-prediction databases were used to identify the potential microRNA to regulate FZD6 expression in BC. Results showed that 60% of the BC samples had a low expression pattern while 40% showed a higher expression level. FZD6 expression analysis showed a significant correlation with tumour invasion ($p < 0.05$), and borderline significance with tumour grade ($p = 0.07$). FZD6 expression showed a highly significant association with the BC patients' survival outcomes. This was mainly due to the overall patients' cohort where tumours with FZD6 elevated expression showed higher recurrence rates (DFS, $p < 0.0001$, log-rank) and shorter survival times (DSS, $p < 0.02$, log-rank). Interestingly, the FZD6 prognostic value was more potent in younger BC patients as compared to those with late

onset of the disease. TargetScan microRNA target-prediction analysis and validated by miRabel showed that FZD6 is a potential target for a considerable number of microRNAs expressed in BC. The current study demonstrates a potential prognostic role of FZD6 expression in young BC female patients and provides a better understanding of the involved molecular silencing machinery of the Wnt/FZD6 signalling. Our results should provide a better understanding of FZD6 role in BC by adding more knowledge that should help in BC prevention and theranostics.

Keywords: cancer screening, Frizzled-6, prognosis, breast cancer, IPA, microRNA, targetscan, Mirabel

INTRODUCTION

Epidemiological and observational studies have reported a transition in the trend of the leading major cause of death from cardiovascular diseases to cancer (Hastings et al., 2018; Stringhini and Guessous, 2018). The main reason for such transition is the improvement in early prediction, diagnosis, and treatment of cardiovascular diseases. This raises pressing needs for more research focusing on the early detection of cancer. Invasive female breast cancer (BC) is the most common cancer type and is considered the main cause of death in women every year with approximately 682,000 cases in 2020 (Sung et al., 2021). BC initiation and progression are controlled by a crosstalk of complex regulatory signalling networks which are not yet fully understood. Among these are Wnt, FGF, Notch, Sonic Hedgehog, and BMP signalling (Katoh, 2017).

Wnt ligands and their receptors, Frizzleds (FZDs), are crucial signalling molecules that play a major role in regulating cellular behaviour and gene transcription during embryonic development and in cancer (Wong et al., 2002; Brennan and Brown, 2004; Turashvili et al., 2006; Ueno et al., 2013). This includes proliferation, differentiation, migration, and aggregation (Turashvili et al., 2006). So far, 19 wnt ligands and 10 FZD receptors have been identified in humans. Interestingly, the first two Wnt members (Int-1 and Int-2) were primarily discovered as oncogenes in mouse mammary tumours (Peters et al., 1984; Mester et al., 1987). Expression of at least eight Wnt ligands (Wnt-2, −3, −4, −5A, −7B, −10B, −13, and −14) and several frizzled receptors was reported in different types of liquid and solid tumours including BC (Howe and Brown, 2004; Zhan et al., 2017; Martin-Orozco et al., 2019; Koni et al., 2020; Wu et al., 2020).

During embryonic development, Wnt members expression was shown to play crucial role in the maintenance or specification of the mammary stem cells (Cantilena et al., 2011) and gland ductal formation (Lin et al., 1992; Brennan and Brown, 2004). Interestingly, this expression was found either upregulated or downregulated in cancer (Boras-Granic and Wysolmerski, 2008; Incassati et al., 2010; Yu et al., 2016) indicating that these members could play a dual role in development and cancer. Therefore, they could be potential therapeutic targets in different types of cancer (Yang et al., 2011; Xie et al., 2018; Zeng et al., 2018). Wnt/PCP (planar cell polarity) signalling, which controls the distribution of Wnt/β-catenin in filopodia protrusions (cytonemes), was shown to regulate cancer cell growth by regulating these cytonemes (Mattes et al., 2018; Fereres et al., 2019). This suggests that inhibiting or manipulating Wnt function could lead to identifying potential cancer therapeutic targets. A number of FZD receptors are being tested for antibody therapeutics including FZD1, 2, 5, 7, and 8 in patients with Wnt driven cancers (reviewed in (Katoh, 2017). A better understanding of the functioning of the Wnt/FZDs signalling mechanism is still needed to determine which component(s) should be targeted for efficient biomarker discovery and targeted therapy.

Among the FZD members that have received very little attention in BC is FZD6, despite its important role in other types of cancer including cervical cancer (Wang et al., 2021), colon (Vincan and Barker, 2008; Xu et al., 2019), leukaemia (Wu et al., 2009; Yuan et al., 2019; Cassaro et al., 2021), hepatocarcinoma (Bengochea et al., 2008), squamous cell sarcoma and adenomas (Haider et al., 2006), oral squamous cell carcinoma (Putnová et al., 2021; Sung et al., 2021), neuroblastoma (Cantilena et al., 2011), glioblastoma (Zhang et al., 2021), pancreatic adenocarcinoma (Yang et al., 2019; Li et al., 2021), and prostate cancer (Saramäki et al., 2006; Han K. et al., 2018). Hence, FZD6 was suggested as a promising therapeutic cancer target (Han K. et al., 2018; Zeng et al., 2018; Patel et al., 2019).

Due to the critical role and importance of the Wnt/FZD signalling function as well as the promise of FZD6 as a therapeutic target, we tailored this study to assess FZD6 protein expression in Saudi female BC aiming at unravelling the correlation of its expression pattern with the clinicopathological features and the survival outcome. We, in addition, analysed the possible potential interactions of FZD6 with several microRNAs known to be expressed in BC to further understand their molecular involvement in the biological complexity of the BC.

PATIENTS AND METHODS

Ethical Approval

All patients included in this study provided written informed consent. The study was reviewed and approved by the Center of Excellence in Genomic Medicine Research (CEGMR) ethical committee (Approval no. 08-CEGMR-02-ETH). Patients' samples collection was carried out according to the guidelines of King Abdulaziz University Hospital, Jeddah, Saudi Arabia.

Breast Cancer and Lymph Nodes Tissue Biopsies

Four hundred and five (405) informed consent Saudi BC female patients only diagnosed with invasive ductal

carcinoma, admitted for surgery and their clinicopathological data were available at the Department of Pathology, King Abdulaziz University Hospital, Jeddah, Saudi Arabia were included for this study. Only one sample per patient was included in the analysis of this study. Patients who received neoadjuvant therapy were excluded from the study. The BC tissue and lymph nodes biopsies of these patients were immediately formalin-fixed after surgery then processed for the standard FFPE (formalin-fixed, paraffin-embedded) blocks. These were used to make tissue microarray (TMA) slides according to the previously reported protocol (Kononen et al., 1998). Briefly, BC tissue cores were punched from donor block(s) in an automated TMA instrument (TMA Master 1.14 SP3 from Histech Ltd. Budapest, Hungary) and inserted into a recipient paraffin block.

Immunohistochemistry

Immunohistochemistry (IHC) was carried out by following the manufacturer's instructions of the automated Benchmark XT slide staining system (Ventana Medical Systems, United States). Briefly, microarray tissue sections were deparaffinized and the antigen was retrieved by cell conditioning buffer (CC1). Anti-FZD6 primary antibody (Abcam ab150545, rabbit polyclonal, 1:100 dilution) was applied manually for 30 min at room temperature. This was followed by several buffer washes and serum blocking. Colour was developed according to the manufacturer's instructions of the Dako Real Detection System (Catalogue number: K5001) which was followed by counterstaining with Hematoxylin. Sections were dehydrated by an ascending series of EtOH, cleared in Xylene, and mounted with DPX-mounting media. FZD6 expression was blindly scored in relation to the patients' clinical data. Placenta tissue was used as a positive control for FZD6 expression analysis.

FZD6 Expression Immunohistochemistry Scoring

FZD6 protein expression of all BC samples was assessed using a Nikon light microscope at ×40 magnification in a blind fashion and compared to the clinicopathological parameters of the patients. Blind IHC scoring was carried out by two independent expert pathologists using the well-known and validated IHC Index Score System (Lipponen and Collan, 1992) without any prior knowledge about the patients' samples, and/or clinical features. The intensity of IHC staining was classified into four categories as follows: level (0): negative or no detectable FZD6 staining; level (1): weak expression, but staining can be detected; level (2): moderate expression, clearly positive but still weak; level (3): strong to very strong expression. Both intensity and the fraction of positively stained cells were used to calculate the staining index score by the following the formula: I = 0xf0 + 1xf1 + 2xf2 + 3xf3; where (I) is the staining index and (f0 to f3) are the fractions of the cells showing the level of staining intensity (from 0 to +3) as previously reported (Lipponen and Collan, 1992; Buhmeida et al., 2008).

Statistical Analysis

Statistical analyses were performed using the SPSS® software packages (version 19). Frequency tables were analysed using the Chi-square test to assess the significance of the correlation between the FZD6 protein expression and the clinicopathological features. Univariate survival analysis using Kaplan-Meier method was performed to calculate the disease-free survival (DFS) and disease-specific survival (DSS). Tests with $p < 0.05$ were considered statistically significant.

Ingenuity Pathway Analysis and microRNA Target-Prediction Analysis

Ingenuity pathway analysis (IPA) software (Qiagen, United States) (http://www.ingenuity.com) has a backend next-generation knowledge base with clarified up-to-date scientific findings from publications, various databases, and related resources (Abu-Elmagd et al., 2017; Jafri et al., 2019; Bahlas et al., 2020; Abou-Elhamd et al., 2021). Here, we used the IPA to perform the core analysis to functionally annotate the genes regulated by FZD6 in BC to identify specific canonical pathways, unique non-directional gene networks, novel molecular signatures, and regulation of cellular, molecular, and bio-functions using the right-tailed Fisher Exact Test and Benjamini Hochberg Correction (BHC) for multiple testing ($p < 0.05$) (Fisher, 1925; Benjamini and Hochberg, 1995; Benjamini and Yekutieli, 2001). Besides, Molecular Activity Predictor (MAP) tool in IPA was used to predict the upstream and downstream effects of either activation or inhibition of molecules regulated by FZD6.

TargetScan is a bioinformatics tool that predicts microRNA targets based on the presence of sites that match the seed region of each miRNA. The microRNAs expressed in BC and obtained by the IPA were validated for FZD6 and WNT ligands target prediction using TargetScanHuman (Version 7.2, http://www.targetscan.org/vert_72/) (Lewis et al., 2005). The microRNAs predicted to target FZD6 by TargetScanHuman were further validated by miRabel microRNA target-prediction platform (Quillet et al., 2019) (http://bioinfo.univ-rouen.fr/mirabel/). miRabel combines four microRNA target-prediction platforms (miRanda, PITA, SVmicrO, and TargetScan) into one easy-to-use database.

RESULTS

Expression Pattern Profile of FZD6 in the Breast Cancer Microarray Tissue Samples

Our results showed that the cellular localization of FZD6 protein expression was mainly cytoplasmic in both primaries and lymph node metastasis tissue samples. About 39% of our samples showed a moderate/strong (high) expression pattern, while most of the samples (61%) showed either a negative or weak (low) expression profile (**Figure 1**). On the other hand, the cytoplasmic expression pattern of BC primaries and their lymph node metastasis sites are illustrated in **Figures 2** and **3** respectively.

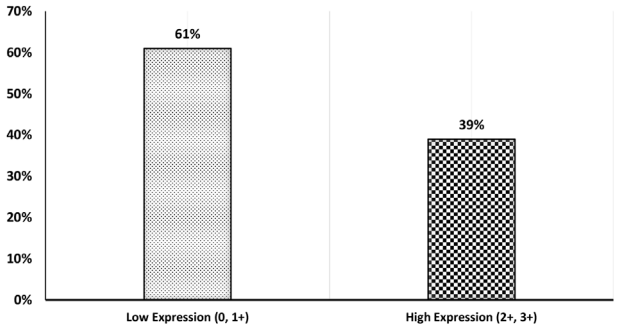

FIGURE 1 | BC patients' cohort distribution according to FZD6 protein expression pattern with low expression (0, 1+) versus high expression (2+, 3+).

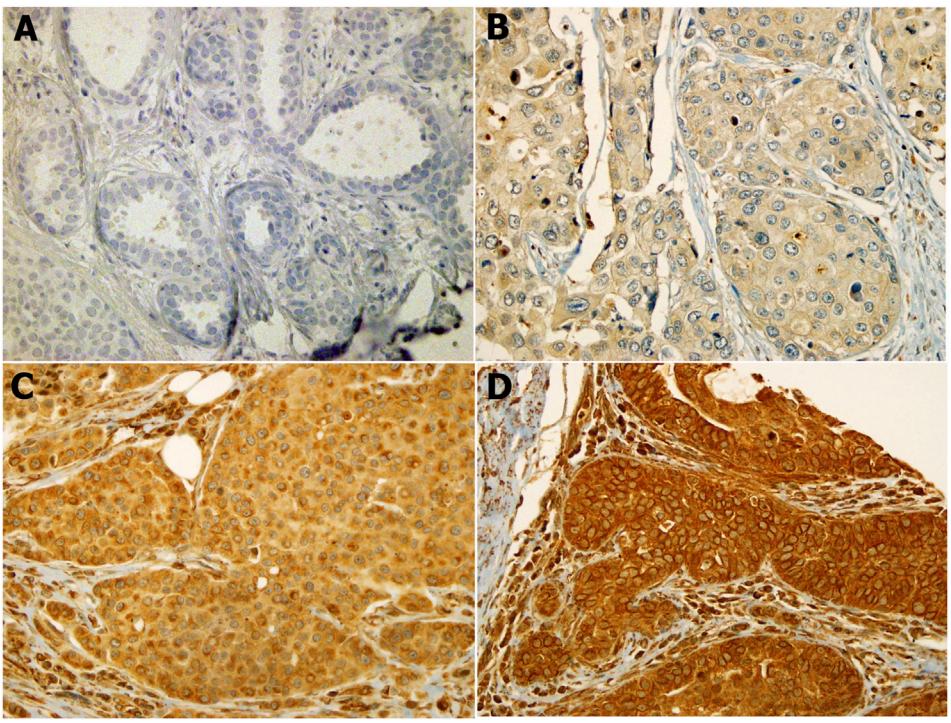

FIGURE 2 | FZD6 cytoplasmic expression pattern in Saudi breast cancer patients is categorised as four levels: **(A)**. Level 1: no expression, **(B)**. Level 2: weak expression, **(C)**. Level 3: moderate expression, **(D)**. Level 4: strong expression.

Correlation of FZD6 Protein Expression Pattern With the Clinicopathological Features

The correlation of FZD6 protein expression with the patients' clinicopathological characteristics using different cut-offs showed that low FZD6 protein expression pattern profile (0, 1+) versus high level of expression (2+, 3+) cut-off (low expression vs high expression) was the most powerful discriminator.

Based on the above-mentioned powerful discriminatory cut-off point, our study showed that there was a significant association between FZD6 protein expression profile and the age of the patients at the time of the diagnosis. BC tissue samples of older patients expressed more FZD6 protein than the tissue of younger patients ($p < 0.05$). Also, a significant correlation was observed between FZD6 expression profile and the tumour invasion property. BC tissues with high invasiveness character expressed more FZD6 protein than less invasive tumours ($p < 0.003$).

Moreover, a significant correlation between FZD6 protein expression pattern and tumour grade ($p = 0.04$) was observed. In fact, tumours with high grade (poorly differentiated cells character) showed higher FZD6 expression pattern as compared to well and moderately differentiated tumour cells ($p = 0.04$). Interestingly, our study cohort revealed a

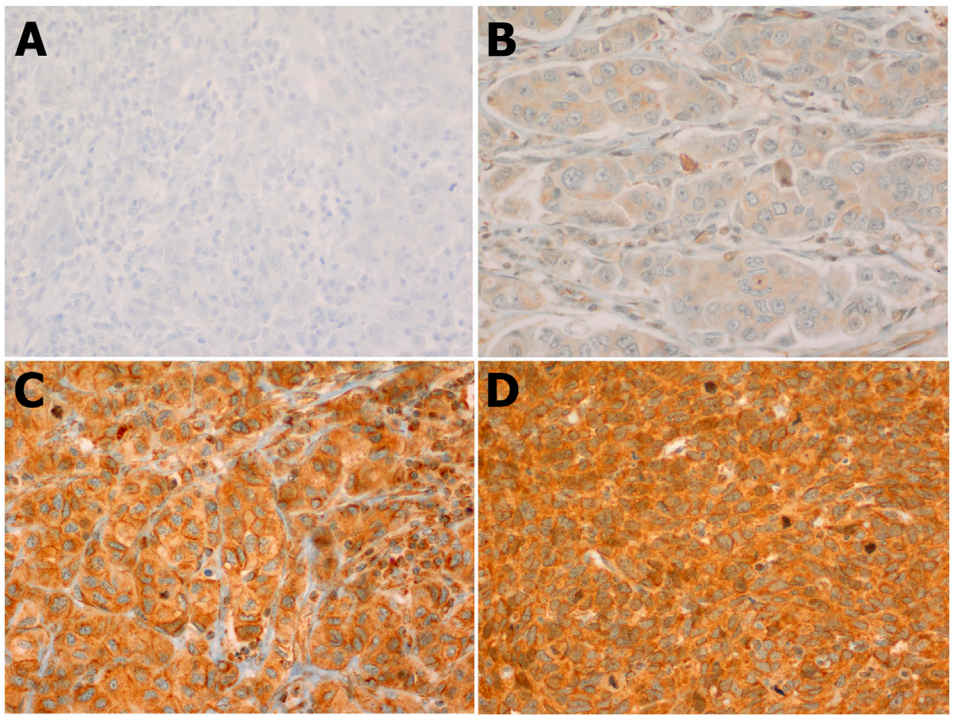

FIGURE 3 | FZD6 cytoplasmic expression pattern in the lymph nodes of Saudi breast cancer patients classified in 4 levels: **(A)**. Level 1: No expression, **(B)**. Level 2: Weak expression, **(C)**. Level 3: Moderate expression, **(D)**. Level 4: Strong expression.

highly significant relationship between the expression profile of FZD6 protein and the incidence of disease recurrence. About 68% of patients with a low FZD6 protein expression profile did not experience any recurrence compared to only 32% of their counterparts with a high FZD6 expression profile ($p < 0.04$). However, the other clinicopathological features did not show any significant correlation with FZD6 protein expression profiles including lymph node status ($p = 0.3$), tumour size ($p = 0.4$), vascular invasion ($p = 0.6$), hormonal status ($p = 0.2$) and HER2 protein expression profile status ($p = 0.3$) (**Table 1**).

Correlation of FZD6 Protein Expression Profile With the Survival Outcomes

Kaplan-Meier survival analysis on the overall cohort showed that BC patients with high FZD6 protein expression patterns experienced a high disease recurrence rate [disease-free survival (DFS)] as compared to those with low expression profiles. For example, at 5 years follow up time, 50% of BC patients with higher FZD6 expression had disease recurrence compared to only 17% disease recurrence rate for the BC patients with low FZD6 protein expression (**Figure 4**, $p < 0.0001$, log-rank).

The assessment of the disease-specific survival (DSS) in the overall cohort using the same cut-off points showed the same trend. This shows that BC patients with their samples expressing weak FZD6 protein expression lived longer. At 5 years follow up time, about 67% of BC patients who had tumours with high FZD6 expression died compared to only 27% death rate for those with a low FZD6 expression pattern (**Figure 5**, $p < 0.02$, log-rank).

To investigate the age-related prognosis value of FZD6, we used 50 years as an age cut-off to split our patient cohort into a young group (up to 50 years) and older (>50 years). Remarkably, we noticed that FZD6 prognosis power to predict the disease recurrence is far stronger in younger BC patients ($p < 0.0001$; log-rank) compared to their matched older patients ($p = 0.5$; log-rank).

Multivariate Cox regression analysis revealed that FZD6 expression pattern profile (low vs. high) in relation to the patients' age, lymph node status, tumour grade, and vascular invasion was an independent poor survival factor for the DFS ($p < 0.04$) but not the DSS ($p = 0.1$).

WNT/FZD6 and miRNA Signalling Pathway Analysis Using IPA, TargetScan, and miRabel

We used ingenuity pathway analysis to dissect FZD6 molecular signalling involved in BC. First, we identified the main Wnt signalling components in BC, among which FZD6 was associated (**Table 2**). The expected expression level (either up or down) of each Wnt molecule in BC was also shown. Our analysis showed that FZD6 is implicated in several signalling

TABLE 1 | Correlations between FZD6 protein expression and BC patients' clinicopathological features.

Clinicopathological feature	Number of cases (%)	FZD6 Expression pattern		*p*-Value
		Low (0, 1+)	High (2+, 3+)	
Age				**0.04**
1=<50	204 (51%)	127 (62%)	77 (38%)	
2 > 50	200 (49%)	119 (60%)	81 (40%)	
Missing data	1 (0%)			
Tumour Invasion				**0.003**
Negative	9 (2%)	5 (56%)	4 (44%)	
Positive	368 (91%)	230 (63%)	138 (37%)	
Missing data	28 (7%)			
(ER + *ve*, PR + *ve*) *vs.* (ER -*ve*, PR -*ve*)				0.22
ER-*ve*, PR-*ve*	105 (26%)	65 (62%)	40 (38%)	
ER + *ve*, PR + *ve*	178 (44%)	97 (55%)	81 (45%)	
Missing data	122 (30%)			
(ER + *ve*, PR -*ve*) *vs.* (ER -*ve*, PR + *ve*)				0.48
ER-*ve*, PR + *ve*	25 (6%)	16 (64%)	9 (36%)	
ER + *ve*, PR-*ve*	50 (12%)	36 (72%)	14 (28%)	
Missing data	330 (82%)			
HER2 -*ve* = 0, HER2+*ve* = 1, HER2 *borderline* = 2				0.32
Negative	193 (48%)	110 (57%)	83 (43%)	
Positive	123 (30%)	77 (63%)	46 (37%)	
Missing data	89 (22%)			
Triple Negative and Triple Positive				0.63
TN	51 (13%)	26 (51%)	25 (49%)	
TP	65 (16%)	36 (55%)	29 (45%)	
Missing data	289 (71%)			
Lymph Node Status				0.35
Negative	123 (30%)	69 (56%)	54 (44%)	
Positive	222 (55)	136 (61%)	86 (39%)	
Missing data	60 (15%)			
Vascular Invasion				0.59
Negative	172 (43%)	101 (59%)	71 (41%)	
Positive	123 (30%)	76 (62%)	47 (38%)	
Missing data	110 (27%)			
Tumour Margin				0.67
Negative	319 (79%)	159 (61%)	124 (39%)	
Positive	53 (13%)	34 (64%)	19 (36%)	
Missing data	33 (8%)			
Tumour Size				0.46
0-3	142 (35%)	83 (58%)	59 (42%)	
3-6	178 (44%)	107 (60%)	71 (40%)	
>7	42 (10%)	29 (69%)	13 (31%)	
Missing data	43 (11%)			
Tumour Grade				**0.04**
Grade 1	61 (15%)	33 (64%)	22 (36%)	
Grade 2	180 (44%)	121 (67%)	59 (33%)	
Grade 3	105 (26%)	55 (52%)	50 (48%)	
Missing data	59 (15%)			
Recurrence				**0.03**
Yes	57 (14%)	30 (53%)	27 (47%)	
No	144 (36%)	99 (69%)	45 (31%)	
Missing data	204 (50%)			
Status at End Point				0.29
Died	38 (9%)	24 (63%)	14 (37%)	
Alive	70 (17%)	51 (73%)	19 (27%)	
Missing data	297 (73%)			

Significant p-*values are indicated in bold.*

pathways including BC, Wnt, cancer-related (such as the epithelial-mesenchymal transition (EMT)), and basal cell carcinoma signalling pathways (**Table 3**). The IPA analysis also showed that FZD6 is implicated in breast adenocarcinoma, and ductal breast carcinoma (**Table 4**). The analysis revealed a considerable number of genes and microRNAs in these types of BC that could be potentially interacting with FZD6 (**Table 4).**

A considerable number of microRNA have been shown to orchestrate many biological processes during embryonic

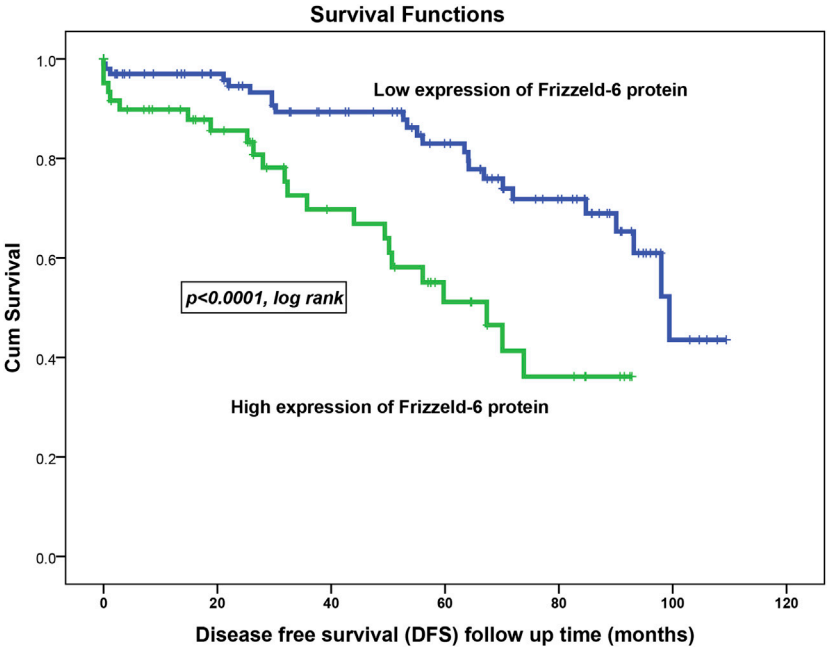

FIGURE 4 | FZD6 overexpression in the overall cohort as a general poor prognosticator for disease-free survival (DFS) of breast cancer patients. During all follow-up period, the recurrence risk factor in patients with low FZD6 expression was 22% compared to 37% in their counterparts with high FZD6 expression.

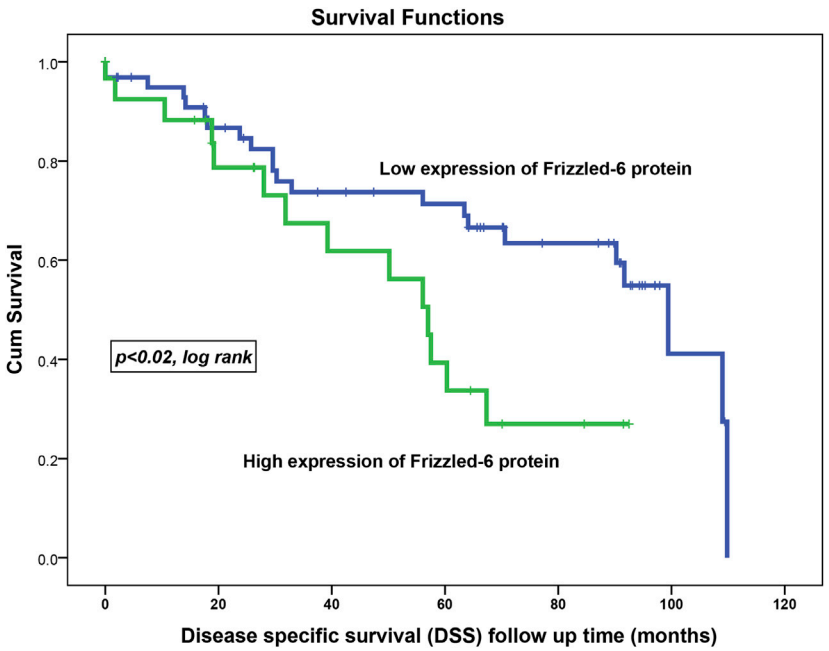

FIGURE 5 | FZD6 overexpression in the overall cohort as a general poor prognosticator for disease-specific survival (DSS) of breast cancer patients. The death risk during all follow-up periods was 34% in patients with low FZD6 expression compared to 47% in their counterparts with high FZD6 expression.

development, adulthood, and in diseases through a gene silencing machinery. We first identified the microRNAs expressed in the BC using the IPA then validated these for target prediction using the TargetScan platform to specifically identify those targeting FZD6 and WNT ligands. At least 30 potential microRNAs that could either directly or indirectly fine-tune or silence FZD6 expression in BC were identified. The 30 microRNAs were further validated

TABLE 2 | Wnt signalling components in breast cancer identified by ingenuity pathway analysis. This shows the expected expression level and cellular location of each WNT signalling component.

Wnt component/Symbol	Entrez gene name	Expected Expression level	Cellular location
CTNNB1	Catenin beta-1	Up	Nucleus
DKK1	Dickkopf WNT signaling pathway inhibitor-1	Down	Extracellular Space
DVL1	Dishevelled segment polarity protein-1	Up	Cytoplasm
DVL2	Dishevelled segment polarity protein-2	Up	Cytoplasm
DVL3	Dishevelled segment polarity protein-3	Up	Cytoplasm
FZD6	Frizzled class receptor-6	Up	Plasma Membrane
GNAQ	G-protein subunit alpha q	Up	Plasma Membrane
SFRP1	Secreted frizzled related protein-1	Down	Plasma Membrane
SFRP2	Secreted frizzled related protein-2	Down	Plasma Membrane
TCF7L2	Transcription factor 7 like-2	Up	Nucleus
TP53	Tumor protein p53	Up	Nucleus
WNT16	Wnt family member-16	Up	Extracellular Space
WNT5A	Wnt family member-5A	Up	Extracellular Space

TABLE 3 | Molecular signalling pathways identified by the ingenuity pathway analysis showing the highest scoring pathways in which FZD6 and other molecules are interacting. These pathways mainly include Wnt signalling, breast cancer, and cancer-related pathways.

Molecular signalling pathway	-log (*p*-value)	Molecules involved
Wnt/β-catenin Signaling	13.1	CTNNB1, DKK1, **FZD6**, GNAQ, SFRP1, SFRP2, TCF7L2, TP53, WNT16, WNT5A
Basal Cell Carcinoma Signaling	8.92	CTNNB1, **FZD6**, TCF7L2, TP53, WNT16, WNT5A
Regulation of the Epithelial-Mesenchymal Transition (EMT) Pathway	7.76	CTNNB1, **FZD6**, HRAS, PIK3CA, TCF7L2, WNT16, WNT5A
Regulation of the Epithelial Mesenchymal Transition in Development Pathway	6.71	CTNNB1, **FZD6**, TCF7L2, WNT16, WNT5A
Breast Cancer Regulation by Stathmin1	3.57	**FZD6**, GNAQ, HRAS, miR-101, PIK3CA, TP53

TABLE 4 | Ingenuity pathway analysis showing FZD6 is implicated in different types of breast cancer. These include breast cancer in general, basal adenocarcinoma, and ductal breast carcinoma. The analysis also identified other important interacting molecules in each cancer/breast cancer type.

Disease/Function	*p*-Value	Molecules/Genes involved
Breast cancer	2.1E-23	PTEN, NFATC2, WNT5A, FANCC, miR-199a-5p (and other miRNAs w/seed CCAGUGU), SFRP1, DKK1, miR-374c-5p (and other miRNAs w/seed UAAUACA),TP53,miR-145-5p (and other miRNAs w/seed UCCAGUU), FOS, GNAQ, PIK3CA, ITGB1, WLS, **FZD6**, SFRP2, TCF7L2, miR-19b-3p (and other miRNAs w/seed GUGCAAA), miR-16-5p (and other miRNAs w/seed AGCAGCA), miR-103-3p (and other miRNAs w/seed GCAGCAU), CTNNB1, LMO2, miR-96-5p (and other miRNAs w/seed UUGGCAC), mir-101,WNT16, miR-21-5p (and other miRNAs w/seed AGCUUAU), NPTX2, ELAVL1, RB1, HRAS, miR-22-3p (miRNAs w/seed AGCUGCC), OGA.
Breast adenocarcinoma	6.35E-14	miR-16-5p (and other miRNAs w/seed AGCAGCA), PTEN, NFATC2, miR-103-3p (and other miRNAs w/seed GCAGCAU), FANCC, CTNNB1, WNT16, TP53, miR-21-5p (and other miRNAs w/seed AGCUUAU), FOS, GNAQ, PIK3CA, ITGB1, **FZD6**, TCF7L2, RB1, HRAS, miR-19b-3p (and other miRNAs w/seed GUGCAAA)
Ductal breast carcinoma	7.64E-12	miR-16-5p (and other miRNAs w/seed AGCAGCA), PTEN, NFATC2, miR-103-3p (and other miRNAs w/seed GCAGCAU), WNT16, TP53, miR-21-5p (and other miRNAs w/seed AGCUUAU), FOS, GNAQ, PIK3CA, ITGB1, **FZD6**, TCF7L2, RB1, HRAS, miR-19b-3p (and other miRNAs w/seed GUGCAAA)

using four platforms (miRanda, PITA, SVmicrO, and TargetScan) that are combined in one (miRabel) microRNA prediction database. First, we pulled out all possible predicted microRNA to interact with FZD6 then blasted 30 microRNA confirmed by the TargetScan. The results confirmed that 29 out of 30 microRNAs predicted by TargetScan were also predicted to target FZD6 by miRabel, only has-miR-302a-b3p was not predicted (**Table 5**) (**Figure 6**) (**Supplementary Table S1**).

DISCUSSION

The word 'cancer' is still horrifying to many, but according to WHO it should not be a death sentence to the cancer patient. This will not be accomplished unless we have globally full control of the disease incidence. One way to achieve this is by identifying the cancer inducers using *in vivo* screening and by discovering new biomarkers that could help us in early diagnosis, prognosis, and therapies. The current study is a part of an OncoScreen project

TABLE 5 | MicroRNAs identified by the ingenuity pathway analysis and validated by TargetScan and miRabel for the microRNA target prediction analysis showing the potential microRNAs expressed in different types of breast cancer and potentially targeting FZD6. The analysis also shows the potential predicted Wnt ligands that could bind to FZD6.

Target rank	miRNAs expressed in breast cancer and FZD6 is a predicted target	Target score	Transcript variants accession of FZD6 (gene ID: 8323) as the predicted target for the miRNA	IPA predicted WNT ligand for the miRNA
1	hsa-miR-101-3p	99	FZD6 (NM_001317796)	WNT2B, WNT7A
2	hsa-miR-302b-3p	98	FZD6 (NM_001164615)	WNT9A, WNT9B
3	hsa-miR-302d-3p	98		
4	hsa-miR-372-3p	98		
5	hsa-miR-373-3p	98		
6	hsa-miR-520c-3p	98		
7	hsa-miR-519a-3p	97		WNT5B, WNT8B
8	hsa-miR-519b-3p	97		WNT5A, WNT8B
9	hsa-miR-568	96	FZD6 (NM_001317796)	WNT2B, WNT3 WNT5A, WNT5B WNT9A, WNT10A WNT16
10	hsa-miR-545-3p	96		WNT5A, WNT5B WNT7A, WNT9B
11	hsa-miR-130a-3p	95	FZD6 (NM_001164615)	WNT1, WNT2B
12	hsa-miR-130b-3p	95		WNT10A
13	hsa-miR-301a-3p	95		
14	hsa-miR-301b-3p	95		
15	hsa-miR-454-3p	95		
16	hsa-miR-3121-3p	94	FZD6 (NM_001317796)	WNT1, WNT2B WNT5A, WNT5B WNT8B, WNT9A WNT9B, WNT11 WNT16
17	hsa-miR-19a-3p	92	FZD6 (NM_001164615)	WNT1, WNT3
18	hsa-miR-19b-3p	92		WNT10A, WNT7B
19	hsa-miR-548l	76	FZD6 (NM_001317796)	WNT5A, WNT8B WNT16
20	hsa-miR-15a-5p	72	FZD6 (NM_001164615)	WNT2B, WNT3A
21	hsa-miR-15b-5p	72		WNT10B
22	hsa-miR-16-5p	72		
23	hsa-miR-195-5p	72		
24	hsa-miR-424-5p	72		
25	hsa-miR-497-5p	72		
26	hsa-miR-30a-3p	69	FZD6 (NM_001317796)	WNT1, WNT2
27	hsa-miR-30e-3p	69		WNT2B, WNT3 WNT4, WNT5A WNT9B
28	hsa-miR-32-3p	69		WNT2B, WNT5A WNT7B, WNT9A WNT10B, WNT16
29	hsa-miR-4677-3p	62		WNT4, WNT5A WNT7A, WNT9A WNT9B, WNT16

aiming at identifying the cancer inducers as well as early cancer biomarkers.

In Saudi Arabia, BC is the leading cancer type with an incidence of 29.7% in women in 2018. Wnt ligands signal to frizzleds mainly through either canonical (β-Catenin activation-dependent) or non-canonical (Wnt or β-Catenin independent) signalling pathways. The Wnt receptor FZD6 has received, as far as we know, nearly no attention to its role in BC except a very few studies (Vouyovitch et al., 2016; Corda et al., 2017; Poodineh et al., 2020). Remarkably, FZD6 has been reasonably studied in other cancer types such as oral (Putnová et al., 2021), prostate (Vatansever et al., 2014), thyroid (Deng et al., 2015), pancreatic adenocarcinoma (Li et al., 2021), osteosarcoma (De Sá Rodrigues et al., 2017), and leukemia (Wu et al., 2009).

In the current study, we showed that elevated FZD6 expression is strongly associated with the early onset of female Saudi BC patients, tumour invasion, and poor survival outcomes. Most importantly, we showed that a higher FZD6 expression level was significantly associated with the survival outcomes of the BC patients including the recurrence (Disease-Free Survival) and the life expectancy after the primary treatment (Disease-Specific Survival). IPA analysis results showed that FZD6 is implicated in BC, breast adenocarcinoma, and ductal breast carcinoma molecular signalling. MicroRNA TargetScan prediction analysis revealed that

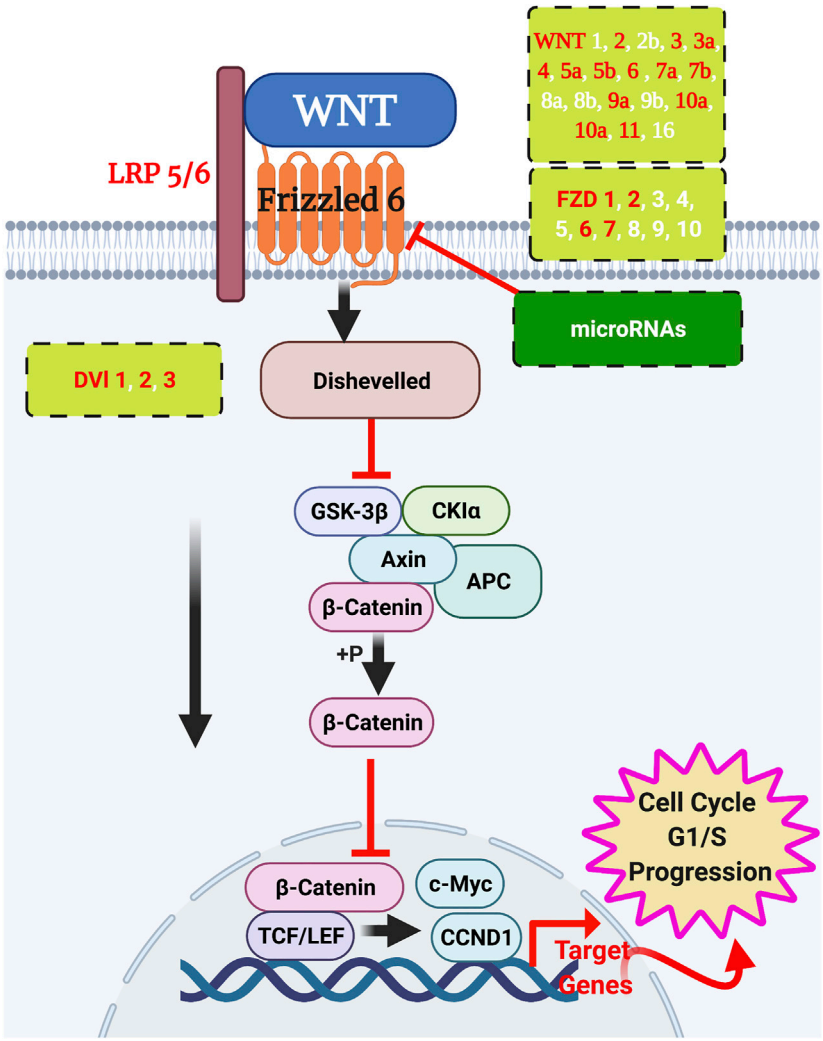

FIGURE 6 | WNT signalling interactions in breast cancer. Wnt ligands signal to their frizzled receptors to activate the downstream cascade of the pathway to initiate the transcription. Validated WNT ligands and frizzled receptors are in red text, and the putative members are in white text. Potential microRNAs to silence the frizzleds' function are listed in **Table 5**. Created by BioRender.

FZD6 is a potential target of 30 microRNAs, however, miRabel microRNA prediction platform validated 29 of these microRNA (i.e. except has-miR-302a-b3p). In triple negative BC (TNBC) cell line, miR-130a-3p was shown to block Wnt signalling components among which was FZD6 (Poodineh et al., 2020). In our IPA and miRabel analysis, we did not see this microRNA targeting FZD6, and hence further studies may be required to confirm this finding.

In Saudi female BC patients, it has been shown that expression of the Wnt axis APC/Axin/DKK3/FRP2/WIF1 was downregulated and not associated with the age of onset of the disease (Khan et al., 2018). It has been previously shown that FZD6 promotes TNBC cell motility and metastasis through the fibronectin-actin axis. This suggested that the noncanonical Wnt signalling is involved in basal-like BC/TNBC progression (Corda and Sala, 2017). It has been suggested that FZD6 through this non-canonical Wnt signalling affects the cell motility and cellular invasion (Corda and Sala, 2017), and hence it is considered an important potential therapeutic target (Van Schie and Van Amerongen, 2020). We reported here that FZD6 is associated with the survival outcomes of BC patients. In liver tumourigenesis, FZD6 was the only frizzled gene found to be associated with tumour recurrence and metastasis (Chen et al., 2018). Besides, the association of FZD6 with tumour invasion reported here is consistent with a previous report showing that WNT11/FZD6 were associated with tumour invasion in colorectal cancer (Gorroño-Etxebarria et al., 2019). The association of FZD6 with the tumour metastatic recurrence that we showed in our current study is also consistent with the previous study mentioned above in TNBC (Corda, 2015). In cervical cancer, silencing FZD6 function caused delayed cellular proliferation, invasion, and EMT transition through HOXC13/WNT5A/FZD6 axis (Tongfei et al., 2021). Similarly in our cohort, cell proliferation, invasion and EMT could be driven by FZD6 elevated expression.

In our IHC analysis, FZD6 did not show a significant correlation with known BC prognostic markers ER/PR or HER2 (**Table 1**). Our knowledge-based IPA analysis did not also show if these markers were 'direct' upstream or master regulators of FZD6. This suggests that FZD6 probably functions 'indirectly' of these markers. In addition, there were some studies that reported some examples of elevated expression of BC biomarkers with either no correlation with ER expression, such as Endoglin (Guo et al., 2017), or not statistically significant in ER-positive BC such as TOX3 protein expression (Han et al., 2016). For the HER2, the worldwide prevalence of its amplification in BCs ranges only between 15 and 30% while in other types of cancer, such as colorectal cancer, HER2 does not show a prognostic value (Kruszewski et al., 2010). The heterogeneity and multiclonality of BC influenced by the population genomic background, patients' lifestyle and the environmental risk factors is making BC a challenging and biologically complex disease with unexpected correlations and outcomes.

Fluorescence recovery after photobleaching (FRAP) approach showed that several Wnt ligands including WNT-1, -2, -3A, -4, -5A, -7A, -9B, and -10B bind to Fzd6 (Kilander et al., 2014). It is always the question of which WNT ligand could initiate FZD6 in BC. The validated function of the Wnt ligands (WNT 2, 3, 3a, 4, 5a, 5b, 6, 7a, 7b, 9a, 10a, 10b, and 11) and their receptors (FZD1, 2, 6, and 7) in BC were recently reviewed in some detail by Xu and his colleagues (Xu et al., 2020). In breast cancer cells and in solid autocrine human growth hormone (hGH) tumours, both WNT4 and its receptor FZD6 were upregulated (Vouyovitch et al., 2016) indicating that WNT4 is a strong Wnt ligand candidate to activate FZD6. Our *in silico* analysis using IPA showed that Wnt5a and Wnt16 are the most aberrant WNT ligands to signal to FZD6 in BC (**Table 3**), however, these results need further validation. It is worth mentioning that Wnt5a/5b are involved in BC invasiveness and metastasis independent of β-catenin signalling (Klemm et al., 2011; Han B. et al., 2018).

The IPA analysis revealed a considerable number of putative microRNAs that could potentially act to silence FZD6 function. We validated these microRNAs using TargetScan and pulled 30 members as potential miRNAs that could target FZD6 function in BC (**Table 5**). We further used miRabel database and validated all these microRNAs except one microRNA. A considerable number of these microRNAs have been shown to have a pivotal role in BC regulation (Tsai et al., 2018). Among these, as an example, is mir-302b, a microRNA we have identified in our IPA analysis, which was shown to target FZD6 in oral squamous cell carcinoma to promote cell invasion and migration (Sun et al., 2021). It was also previously shown that miR-199b-5p targets HER2 in BC (Fang et al., 2013) as well as directly targeting Fzd6 to activate the signalling cascade of Wnt4, β-catenin, Tcf7, and C-myc during thymic aging (Wang et al., 2020).

The above-mentioned findings we reported here suggest a poor prognostic value of FZD6 overexpression in the early onset of BC through probably affecting cell proliferation, EMT, distant metastasis, and by compromising the normal molecular signalling cascade involved in these processes. Further experimental validation of FZD6 master regulators including microRNAs is needed with taking into consideration other published data that are not included in our analysis.

CONCLUSION

The current study is a part of a cancer prevention program called OncoScreen aiming at screening for cancer inducers and identifying biomarkers for early cancer diagnosis and prognosis. Expression pattern analysis of FZD6 in a Saudi BC cohort showed that its elevated expression is associated with tumour invasion, metastasis, and worse survival outcomes mainly in younger patients. Several WNT ligands and microRNAs were shown to potentially regulate FZD6 expression and function. As far as we know, this study is the first to analyse FZD6 expression in female BC Saudi patients and assess its prognostic value.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Center of Excellence in Genomic Medicine Research (CEGMR), King Abdulaziz University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MA-E, AB, MA, and JA-M: study design, histopathological and statistical data analysis, tissue microarray, writing up the manuscript, executing the work, and supervision of the technical staff. MA-E and PNP: *in silico* study analysis and its writing up. HA, AFA, RE, MHA-Z: technical assistance, writing up and revision of the manuscript. MA-E, AB, MA, MR, MIN: data analysis and interpretation, writing up and revision of the manuscript. All authors approved the final version of the manuscript.

FUNDING

The authors extend their appreciation to the Deputyship for Research and Innovation, Ministry of Education in Saudi Arabia for funding this research work through the project number (IFPRC-088-247-2020) and King Abdulaziz University, Deanship of Scientific Research (DSR), Jeddah, Saudi Arabia.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2022.783735/full#supplementary-material

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frontiers in Molecular Biosciences

REVIEW
published: 21 February 2022
doi: 10.3389/fmolb.2022.783450

Breast Cancer; Discovery of Novel Diagnostic Biomarkers, Drug Resistance, and Therapeutic Implications

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Breast cancer is the second most reported cancer in women with high mortality causing millions of cancer-related deaths annually. Early detection of breast cancer intensifies the struggle towards discovering, developing, and optimizing diagnostic biomarkers that can improve its prognosis and therapeutic outcomes. Breast cancer-associated biomarkers comprise macromolecules, such as nucleic acid (DNA/RNA), proteins, and intact cells. Advancements in molecular technologies have identified all types of biomarkers that are exclusively studied for diagnostic, prognostic, drug resistance, and therapeutic implications. Identifying biomarkers may solve the problem of drug resistance which is a challenging obstacle in breast cancer treatment. Dysregulation of non-coding RNAs including circular RNAs (circRNAs) and microRNAs (miRNAs) initiates and progresses breast cancer. The circulating multiple miRNA profiles promise better diagnostic and prognostic performance and sensitivity than individual miRNAs. The high stability and existence of circRNAs in body fluids make them a promising new diagnostic biomarker. Many therapeutic-based novels targeting agents have been identified, including ESR1 mutation (DNA mutations), Oligonucleotide analogs and antagonists (miRNA), poly (ADP-ribose) polymerase (PARP) in BRCA mutations, CDK4/6 (cell cycle regulating factor initiates tumor progression), Androgen receptor (a steroid hormone receptor), that have entered clinical validation procedure. In this review, we summarize the role of novel breast cancer diagnostic biomarkers, drug resistance, and therapeutic implications for breast cancer.

Keywords: diagnostic biomarkers, circular RNAs, miRNAs, drug resistance, breast cancer

OPEN ACCESS

Edited by:
Liaqat Ali,
National University of Medical Sciences (NUMS), Pakistan

Reviewed by:
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Bold Therapeutics, Canada
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Specialty section:
This article was submitted to Molecular Diagnostics and Therapeutics, a section of the journal Frontiers in Molecular Biosciences

Received: *26 September 2021*
Accepted: *02 February 2022*
Published: *21 February 2022*

Citation:
Afzal S, Hassan M, Ullah S, Abbas H, Tawakkal F and Khan MA (2022) Breast Cancer; Discovery of Novel Diagnostic Biomarkers, Drug Resistance, and Therapeutic Implications. Front. Mol. Biosci. 9:783450. doi: 10.3389/fmolb.2022.783450

1 INTRODUCTION

Breast cancer is a kind of cancer that affects mostly females and is a primary factor of mortality worldwide (Wu and Chu, 2021). It is a heterogeneous disease with six distinct molecular subtypes: luminal A (progesterone receptors (PR)+, estrogen receptor (ER)+, Human epidermal growth factor receptor 2 (HER2)-, and Ki67), luminal B (ER+, HER+/−, and Ki67+), human epidermal growth receptor 2(HER2)+, basal-like subtype (ER-, PR- and HER2−) normal breast like and claudin low type (where low expression of cellular adhesion genes can be detected) (Perou et al., 2000). Breast cancer initial detection and monitoring are two significant treatments that enhance therapy results

and provide patients with a positive prognosis (Pace and Keating, 2014). Mammography is a common approach for identifying breast cancer, although it has several drawbacks, such as a low sensitivity of 25%–59% for cancer detection in younger females with dense breasts. It has also recorded erroneous negative and positive findings, as well as a 1%–10% over detection rates (Bleyer and Welch, 2012; Oeffinger et al., 2015).

Biomarkers that aid in the diagnosis, prognosis, and prediction of breast cancer are essential for timely identification and appropriate control of the disease throughout treatment (Hayes et al., 2001; Weigel and Dowsett, 2010). Moreover, an increasing percentage of patients are demanding personalized or unique treatments, demanding the development of novel biomarkers for diagnostic and prognostic procedures as well as intact cells, are utilized as biomarkers in the diagnosis of cancer. Artificial intelligence technologies like machine learning have the potential to greatly enhance the existing anti-cancer medication development process. Constituents produced by cancer-affected cells or various tissues in reaction to tumors, and also physiological indicators that may be recognized by diagnostics or molecular technology, are all examples of cancer biomarkers (Loke and Lee, 2018; Voith von Voithenberg et al., 2019). Biomarkers may be used to assess the biological condition of a disorder, which can then be used to identify the type of the tumor, its progression, or therapy responses, assisting in the control of breast cancer (Wu and Chu, 2021). Because tumor cells are so heterogeneous, a singular biomarker is insufficiently sensitive or precise to effectively diagnose cancer growth and metastasis, hence a combination of biomarkers is preferred. Significant advances in genetic fingerprints and molecular signaling processes have found a variety of biomarkers in tissues and blood (liquid biopsies) that may be used to predict the likelihood of cancer spread, resurgence, therapy recommendations, prediction, and medication tolerance. Some of these biomarkers have been utilized in clinical trials, however, their sensitivity and selectivity are zero (Nalejska et al., 2014; Wu and Chu, 2021). As a result, novel and more effective biomarkers are required. Moreover, several therapeutic approaches for breast cancer are still in their early phases of development, therefore, it is vital to find precise biomarkers that may be used to help with immunotherapies (Wu and Chu, 2021).

2 TYPES OF BIOMARKERS

Larger molecules such as nucleic acids, genetic alterations, and protein molecules, as well as intact cells, are utilized as biomarkers in the diagnosis of cancer. They can be observed in blood in the form of circulating tumor cells, DNA, and RNA enabling liquid biopsies a useful clinical technique (Eccles et al., 2013; Berghuis et al., 2017; Voith von Voithenberg et al., 2019). Prognostic and predictive biomarkers are two categories of biomarkers linked to likely clinical results and therapy success in breast cancer subtypes (Fine and Pencina, 2015; Janes et al., 2015; Simon, 2015).

3 ROLE OF MACROMOLECULES IN BREAST CANCER DIAGNOSIS

3.1 DNA

Alteration in DNA methylation is one of the major significant molecular changes in carcinogenesis. Adenomatous polyposis coli (APC) and retinoic acid receptors-2 (RARb2) methylated promoters were discovered in 93.4% and 95.6% of blood samples from females having breast cancer, respectively, but not in healthy people (Swellam et al., 2015). All methylation variations surpassed the conventional markers CEA and CA 15–3 in detecting early breast cancer, low-grade tumors, and Triple Negative Breast Cancer (NBC). Utilizing a human methylation DNA study BeadChip. Yang et al. revealed that hypomethylation of S100 calcium-binding protein P (S100P) and hyalurono glucosaminidase 2 (HYAL2) in the peripheral blood is linked with breast cancer (Yang et al., 2015; Yang et al., 2017). Both genes with lower methylation have been proven to be possible biomarkers circulating in blood, for the diagnosis of breast cancer, specifically in adolescent girls in the initial stages (Bleyer and Welch, 2012; Fleming and Powers, 2012).

The latest study has found that aberrant DNA methylation is significantly linked to breast cancer, and suggesting that DNA methylation testing can aid in predicting the prognosis of breast cancer patients. MAST1, PRDM14, and ZNF177 irregular DNA methylation variants were found and verified as prospective breast cancer molecular indicators by X Mao et al. X Mao et al. also showed that the DNA methylation range of ADCY4, CPXM1, DNM3, PRDM14, PRKCB, and ZNF177. In essence, these findings point to the development of novel epigenetic prognosis systems that might assist in the detection and prediction of breast cancer therapy (Uehiro et al., 2016). An exceptionally sensitive mobile cell-free DNA (cfDNA) system with epigenetic biomarkers and droplet digital methylation-specific PCR (ddMSP) has been created for the early diagnosis of breast cancer. Ras-specific guanine nucleotide-releasing factor 1 (RASGRF1), carboxypeptidase X (CPXM1), Hox-A10 (HOXA10), and Dachshund homolog 1 (DACH1) were the four methylation markers employed in the efficient screening method (Uehiro et al., 2016). This epigenetic-marker-based technique was able to reliably discriminate women with breast cancer from healthy controls, hinting that it might be utilized for breast cancer screening and therapy (Uehiro et al., 2016).

3.2 Proteins

Circulating protein has been considered as the second choice as a biological marker for the recognition and analysis of Breast cancer (BC). Blood proteomics and mass spectrometry have analyzed the systemic and comprehensive visualization of the blood proteomics pathologically and physiologically, resulting in the finding of numerous protein biomarkers in blood that can be used as effective diagnostic biomarkers in breast cancer detection. A panel of trefoil factor (TFF) 1, TFF2, and TFF3 have been stated as promising biomarkers for BC screening as they can express specific proteins differentially in the serum of BC patients that cannot be produced by healthy cells (Ishibashi et al., 2017). The

two well-known groups such as Cks of intermediate filaments and glycoprotein (MUC) family can produce several classical breast cancer biomarkers. The CA 15–3 assay, for example, is currently used for tracking purposes in treatment (Duffy, 2006), while CKs have been proposed as early-stage BC markers, but their efficacy is masked due to poor sensitivity (Levenson, 2007). The serum epithelial membrane antigen/CK1 concentration ratio is recommended as possible diagnostic marker, especially for initial stage breast cancer diagnosis. The diagnostic capability of this new combination was assessed better than CA 15–3 (Attallah et al., 2014).

Besides, some other proteinaceous biological markers with promising diagnostic capability have been revealed by ELISA. Among these proteins, a single diagnostic marker model of pleiotrophin (PTN) (Ma et al., 2017a), and double diagnostic marker models such as integration of microRNA (miRNA) miR-127-3p with human epididymis secretory protein 4 (HE4) (Lu et al., 2017), human anterior gradient (AGR) 2 with AGR3 (Garczyk et al., 2015) and vascular endothelial growth factor (VEGF) with CA 15–3 (Ławicki et al., 2016). Serum apolipoprotein C-I (apoC-I) has demonstrated promise results in prognosis and diagnosis of triple-negative Breast cancer (TNBC), as it could distinguish TNBC from non-TNBC cases by greater ApoC-I mRNA and protein expression in the former when compared to both non-TNBC affected individuals and controls (Song et al., 2016).

3.3 Autoantibodies

Autoantibodies are reported as another approach used as diagnostic biomarkers with the potential of multiple targets, short time-frames, and minimalist hardware (Soler et al., 2016). The associated antigens of these antibodies are synthesized by the body's cells; in healthy cells, it is expressed at a modest level, while it is overexpressed in malignant cells. The immune system determines this expression, using toll-like receptors (TLRs) for the innate response, thus reverting tumor growth. MUC1, an integral membrane protein, is overexpressed in 90% of adenocarcinomas and has been linked to tumor aggressiveness (Zaenker et al., 2016). Antibodies that target oncogenic and tumor suppressor proteins are considered as the significant diagnostic biomarkers for the efficient detection of breast cancer. The presence of autoantibodies before the medical diagnosis of the disorder in paraneoplastic syndrome and systemic autoimmune diseases has raised the prospect that the medical diagnosis of BC could also be carried out through detection of auto-immunoglobulins (Fernández Madrid, 2005). As a result, extensive research has revealed the detection of tumor-associated antigens by autoantibodies that were detected in the patient blood sample. The integration of modern proteomics, advanced genomics, high-throughput technology, and traditional immunological approaches has significantly aided advancement in this area (Fernández Madrid, 2005).

3.4 miRNA

miRNAs are single-stranded, non-coding, small (20–25 nucleotide) RNAs that suppress the post-transcriptional expression of specifically selected genes via mRNA breakdown or mRNA expression. The detection and persistence of miRNAs in the bloodstream and their role in diagnostic and therapeutic approaches have been studied thoroughly (Schwarzenbach et al., 2011). Since miRNAs are soluble and observable in cancer cells (Shen et al., 2013), blood, plasma, and patients' saliva, they can be used as biological markers for non-invasive early diagnosis, detection, and treatment of breast cancer (Mitchell et al., 2008; Schwarzenbach et al., 2014). miRNAs are released into the bloodstream by apoptotic cells (Schwarzenbach et al., 2011). miRNAs can travel through the bloodstream in two forms: cell-free (Ago2-related) or embedded in membrane vesicles, microvesicles, or exosomes (Simpson et al., 2009). miRNAs profiling studies can classify dysregulated miRNAs and categorize patients of breast cancer for therapies, highlighting their potential to be used as a predictive and therapeutic biomarker (McGuire et al., 2015). Furthermore, like oncogenes (oncomiRNAs) and tumor suppressors, unregulated miRNA is responsible for tumor growth, development, cell death, invasion, and cell proliferation. Recently, numerous suspected candidates, for example, miR-221, miR-21, and miR-145, have appeared in the blood serum or plasma of BC-affected individuals. Blood-based identities containing miR-221 and/or miR-21 have been shown to have higher diagnostic susceptibility than CEA and CA 15–3 for all stages of cancer, distinguishing BC subjects from patients with benign tumors and healthy people and doing better in distinguishing the TNBC subtype from healthy subjects (Gao et al., 2013; Motawi et al., 2016; Thakur et al., 2016). Exosomal miR-21 expression has also been shown to be elevated in Breast cancer patients' plasma-derived exosomes. The exosomal miR-21 and miR-1246 form modest diagnostic (Hannafon et al., 2016).

In terms of diagnosis, Iorio et al. (2005) discovered a 13-miRNA hallmark that could differentiate affected Breast cancer from healthy breast tissues with 100% precision. Blenkiron et al. (2007) discovered 133 miRNAs with abnormal expression patterns in breast tumor tissues irrespective of normal breast tissues. Despite the discovery of miRNAs with abnormal expression in BC tissues, there are still inconsistencies between the variously identified miRNA signatures. Roth et al. introduced single circulating miRNAs as diagnostic and prognostic instruments after discovering miR-155 in the serum of Breast cancer affected individuals but not in stable controls, and Heneghan et al. identified elevated miR-195 activity in the bloodstream of only affected subjects with BC (Heneghan et al., 2010; Roth et al., 2010). Some miRNAs, such as miR-21and miR-29a or 4-miRNA signature, have been found in the plasma of BC patients (miR-222, miR-16, miR-25, and miR-324-3p) (Wu et al., 2011; Hu et al., 2012).

A vast number of other miRNAs have been reported that overexpress in Breast cancer a few examples are miR-29a, miR-146a, miR-373, miR589, miR-221/222 cluster, miR-9, miR10b, miR-96, miR-181, miR-375, and miR-520c. These miRNAs are linked with treatment, diagnosis, and prognosis of Breast cancer (Polytarchou et al., 2012; Khoshnaw et al., 2013; Sandhu et al., 2014).

3.5 CircRNAs as Diagnostic/Prognostic Markers

Circular RNAs (circRNAs) are recently identified non-coding RNAs that are classified as tiny endogenous RNAs with a broad distribution, various forms, and several regulatory applications (Ashwal-Fluss et al., 2014). Sanger et al. (1976) were the first to find cricRNA in the viroids. Up till now, a large number of circRNAs have been found in various cell lines and organisms (Bleyer and Welch, 2012; Pace and Keating, 2014; Oeffinger et al., 2015), namely protozoa, fungi, worms, plants, fish, mice, insects, and humans (Wang et al., 2014; Westholm et al., 2014). CircRNAs are present in large numbers, about 1/8th of the human genome's transcriptome can generate observable circRNAs, and their expression levels are much more than tenfold higher than that of the comparable linear mRNAs (Salzman et al., 2012; Jeck et al., 2013). Furthermore, as a result of their covalent closed-loop configuration and absence of free terminal ends, circRNAs are much more stable unlike linear RNAs, conferring susceptibility to deterioration by endonuclease R (RNase R) (Chen and Yang, 2015). CircRNAs can also be used to distinguish and recognize various tumor types due to their ability to express particular cell types, tissues, and developmental stages, as well as the fact that different subtypes of circRNAs can be generated (Salzman et al., 2013; Smid et al., 2019). Given the above, we conclude that circRNAs have growing research potential. Various biochemical roles of circRNAs have been discovered as science has progressed. CircRNAs may serve as "sponges" for microRNAs (miRNAs), influencing the role of miRNA target genes (Hansen et al., 2013). Furthermore, circRNAs can be attached to various RNA binding proteins (RBPs), influencing the role of the parental genes (Zeng et al., 2017; Kristensen et al., 2019). Surprisingly, provided evidence suggests that circRNAs may express proteins/peptides playing a role in tumor development and progression (Legnini et al., 2017; Zheng et al., 2019). CircRNAs' special properties and biological roles show the role of circRNAs in tumor growth, replication, metastasis, invasion, and drug tolerance, implying that circRNAs can be used as biological markers and tumor therapeutic goals (Liu et al., 2018a; Kun-Peng et al., 2018). Circular RNAs can avoid exonuclease-induced degradation and are more soluble in blood or plasma than linear RNAs because of their closed continuous loop structure (Alhasan et al., 2016). It has been shown that ncRNAs, such as miRNAs and non-coding RNAs, can serve as reliable biological markers for hepatocellular carcinoma (Li et al., 2015). CircRNAs are regaining attention among researchers as high-throughput sequencing and bioinformatics technology improves. Due to their stability and tissue specificity, circRNAs have been established as suitable biological markers for the diagnosis of gastric cancer (Simon, 2015), hepatocellular carcinoma, and other cancers (Qin et al., 2016). Evidence have revealed that circRNAs can cause tumorigenesis, providing a new approach for identifying diagnostic biomarkers (Kulcheski et al., 2016; Zhu et al., 2017).

Using the circRNA microarray method, Lu et al. determined the circRNA expression pattern in breast cancer and normal tissues and discovered that hsa circ 103110, hsa circ 104689, and hsa circ 104821 levels were upregulated in breast cancer cells with area under the curve (AUC) value of 0.63 (0.52–0.74), 0.61 (0.50–0.73), and 0.60 (0.49–0.71) respectively, while hsa circ 006054, hsa circ 100219, and hsa circ 406697 were downregulated with the area under the curve (AUC) value of 0.71 (0.61–0.81), 0.78 (0.69–0.88) and 0.64 (0.52–0.75) respectively. As a result, mixing hsa circ 006054, hsa circ 100219, and hsa circ 406697 yielded successful diagnostic results as mentioned in **table 1** (Lü et al., 2017). Similarly, Yin et al. found that in the plasma of breast cancer patients, 19 circRNAs were upregulated and 22 were downregulated as opposed to stable controls (Maselli et al., 2019). Further investigation found that the plasma hsa circ 0001785 had a higher diagnostic accuracy than CEA and CA15-3. Furthermore, hsa circ 0001785 plasma levels were correlated with histological grade (p = 0.013), TNM stage (p = 0.008), and remote metastasis (p = 0.016), indicating a possible biomarker for breast cancer diagnosis. The expression levels of hsa circ 0001785 were shown to be lower in post-operative BC patients' plasma samples relative to pre-operative patients (Sarkar and Diermeier, 2021).

3.5.1 Limitations of Circulating miRNA as a Diagnostic Biomarker

The development of a precise and effective biomarker for breast cancer diagnostic approaches is challenging at every step ranging from sample collection to data processing (Witwer, 2015). For example, the low abundance of circulating miRNAs as diagnostic biomarker hinders their detection using microarray-based miRNA profiling techniques. Modified strategies can be adopted to reduce the limitations; such as miRNA isolation before expression profiling (Hamam et al., 2016). Another issue is sample collection which can be resolved by serum selection to avoid limitations of excluding a large number of samples. Because recent studies reported a high level of circulating miRNA in serum than in plasma (Wang, 2012). A recent study identified the fluctuations in circulating miRNA levels in response to chemotherapy. This drawback can be eliminated by collecting blood samples before chemotherapy (Diener et al., 2015).

3.6 Exosome

Exosomes are extracellular membrane-bound vesicles that are nano-sized (30–100 nm) and actively released by cancer cells and neighboring cells present in the tumor microenvironment (TME) (Taylor and Gercel-Taylor, 2013; Gajos-Michniewicz et al., 2014). They are surrounded by a lipid bilayer composed up of phosphoglycerides, ceramides, sphingolipids, and cholesterols (Vlassov et al., 2012) and comprise a diverse array of molecules such as DNA, sugars, proteins, peptides, lipids, mRNAs, miRNAs, as well as other types of ncRNAs (Moreno-Gonzalo et al., 2014). Exosomes like miRNAs, are present in a variety of human bodily fluids, including blood, sweat, urine, and breastfeeding (Vlassov et al., 2012; Raposo and Stoorvogel, 2013).

Exosomes can facilitate tumor development, angiogenesis, immunosuppression, and metastasis by promoting intermodulation between tumor cells and healthy or cancer-affected stromal cells

TABLE 1 | Regulation pattern of different circRNAs in cancer lesions.

Regulation pattern	Types of CircRNAs	Value of area under the curve (AUC)
Upregulation in cancer lesions	hsa circ 103110	0.63 (0.52–0.74)
	hsa circ 104689	0.61 (0.50–0.73)
	hsa circ 104821	0.60 (0.49–0.71)
Downregulation in cancer lesions	hsa circ 006054	0.71 (0.61–0.81)
	hsa circ 100219	0.78 (0.69–0.88)
	hsa circ 406697	0.64 (0.52–0.75)

(Alderton, 2012; Moon et al., 2016a). The surface proteins present on circulating extracellular vesicle (EVs), developmental endothelial locus-1 protein (Del-1) (Moon et al., 2016a), and fibronectin (Moon et al., 2016b), are promising materials for cancer detection. Fibronectin, a matrix protein present extracellularly, binds to several integrins and activates a variety of signaling proteins, including FAK, Src, and Akt (Moon et al., 2016b). Fibronectin levels rose dramatically ($p < 0.0001$) throughout all phases of breast cancer and went back to normal after the tumors were removed. The clinical diagnostic efficacy for fibronectin recognition outside the cellular vesicles was better than that in plasma (Moon et al., 2016b). This underscores the significance of extracellular matrix proteins in breast cancer. The high amount of Del-1 in patients' circulating exosomes (p 0.0001) resulted in excellent diagnostic success in distinguishing patients with early-stage breast cancer from the control system (Moon et al., 2016a).

4 ROLE OF BIOMARKERS IN DRUG RESISTANCE

Chemotherapy treatment has a significant role in the prevention of breast cancer recurrence and spreading (Liu et al., 2018b). But the main problem of this method is Chemo-therapeutic resistance, hsa-circ 0006528 is upregulated in breast cancer cells resistant to Adriamycin resistant breast cancer (ADM), presumably by the circ. pathway. Axis of RNA/miR-7-5p/Raf1 (Guo et al., 2020). Low concentrations of miR-7 expression have long been associated with resistance to breast cancer chemotherapy. Another research of ADM-resistant breast cancer discovered that circKDM4C downregulation inhibited tumor proliferation and alleviated ADM resistance by controlling the miR-548p/PBLD axis (Ma et al., 2019; Yang et al., 2020).

Furthermore, the level of expression of circMTO1 (hsa circ 007874) in monastrol-resistant breast cancer cell lines is substantially lower than in monastrol-sensitive breast cancer cell lines, and uncontrolled expression of circMTO1 will reverse monastrol resistance through the circRNA/TNF receptor-associated factor 4 (TRAF4)/Eg5 pathway. Furthermore, Ma et al. discovered that circMOTL1, which could play an important role in breast cancer cell PTX resistance by controlling the AKT pathway, encouraging anti-apoptotic protein expression, and impeding pro-apoptotic protein synthesis, is found to be elevated in breast cancer (Greene et al., 2019). Yang et al. reported that in Breast cancer cells, the expression of circ-ABCB10 is increased. Through the let-7a-5p/DUSP7 axis, Circ-ABCB10 regulates PTX resistance, apoptosis, invasion, and autophagy in breast cancer cells (Wu et al., 2019).

The impact of Erα36 on the oncogenesis of breast and drug resistance was assessed by Pangano et al. (Yin et al., 2018). Tamoxifen, reported as anti-estrogen, has been shown to act as an agonist of ER36 to proliferate, invade, and metastasize breast cancer cells (Yin et al., 2018), which explains why many breast cancer patients develop drug resistance to anti-estrogens that block the signaling pathways mediated by ER36. A serum autoantibody was recently discovered that functions against ERα in a wide proportion of patients affected by breast cancer and was shown to cause ER36, leading to tamoxifen tolerance (Maselli et al., 2016; Maselli et al., 2019).

4.1 Role of miRNA

There are several miRNAs whose expressions were downregulated in drug resistance BC. In BC-affected tissues and cells resistant to taxol, the increased expression of nuclear receptor co-activator 3 (NCOA3) results in decreased expression of miR-17 and miR-20b. This shows that these three NCOA3, miR-17, and miR-20b may function as active biological markers and therapeutic targets in breast cancer resistance to taxol (Ao et al., 2016).

Another study reported the overexpression of miR-18a in TNBC cells patients who had received neoadjuvant paclitaxel, thus inhibiting dicer expression and increasing paclitaxel resistance in TNBC cells (Sha et al., 2016). There are four miRNAs; miR-90b, 130a, 200b, and 452 that can regulate drug-related cellular pathways, thus leading to chemoresistance as shown in **Figure 1** (Jayaraj et al., 2019).

4.2 Role of circRNAs

In adriamycin-resistant cell lines and tissues sensitive to Adriamycin, a high-level expression of hsa_circ_00006528 was discovered by Gao et al. (Ma et al., 2017b) by using circRNA microarray expression profiles. The hsa_circ_00006528-miR-7–5p-Raf1 axis has a regulatory effect on breast cancer resistance to adriamycin. This highlights the possibility of the use of hsa_circ_00006528 in controlling the factor of drug resistance. This concludes that for breast cancer treatment, circRNAs come up with novel and reliable therapeutic strategies (Ma et al., 2017b).

has-circ_00006428, a circular RNA, can be used as a promising therapeutic candidate due to its role in reducing drug resistance. This

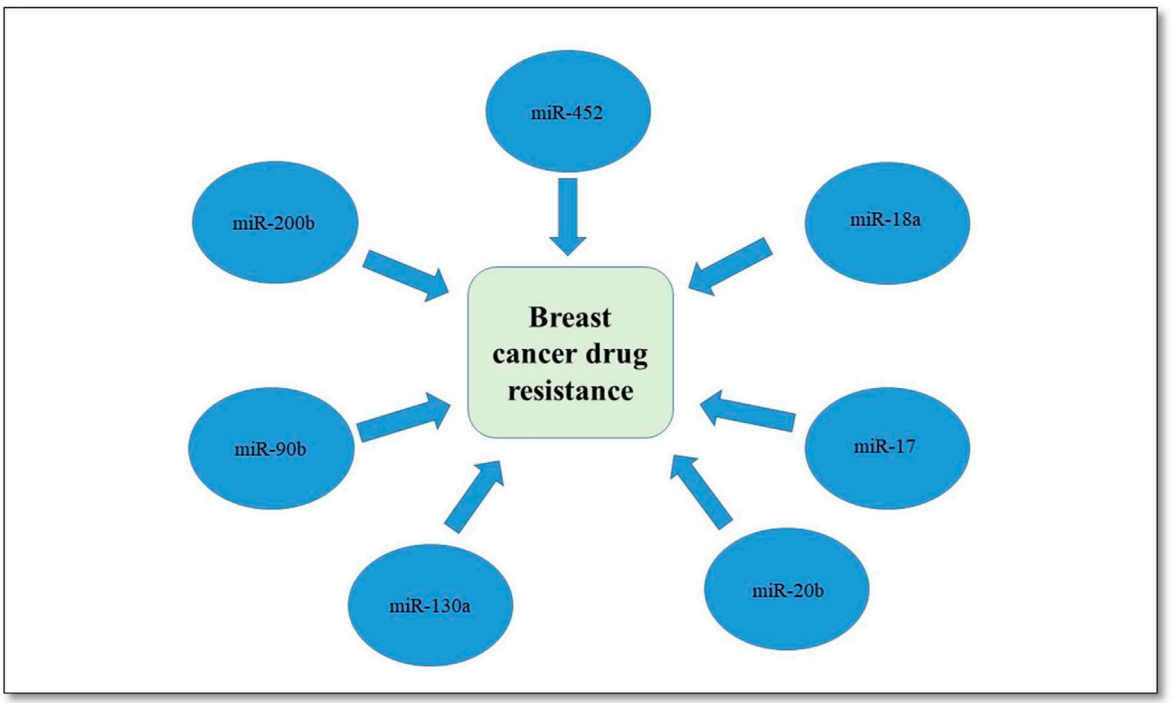

FIGURE 1 | Role of different miRNA in drug resistance.

statement can be supported by its overexpression in Adriamycin-resistant cells while expression in Adriamycin-sensitive cells. Besides, the regulatory role of hsa_circ_00006528-miR-7-5p-Rafl axis in Adriamycin resistant breast cancer was revealed (Ma et al., 2017b).

5 THERAPEUTIC IMPLICATIONS

5.1 CircRNAs as Therapeutic Targets in Breast Cancer

New progress in RNA-based therapies plus aberrant expression of circRNAs in breast cancer makes them more potential therapeutic sites (Lei et al., 2019). One solution may be to create synthetic circRNAs with several binding sites for certain oncogenic proteins or miRNAs that could be inserted exogenously to restore the natural regulatory network in the cell and reduce cancer development (Tay et al., 2015; Liu et al., 2018c). On the other hand, endogenous circRNAs can be useful for the treatment of cancer (Dragomir and Calin, 2018). Any of the tumor-suppressor circRNAs discussed above are prime candidates for further development as therapeutic instruments. CircFOXO3, circCCNB1, circKDM4C, circFBXW, and circTADA2A were all shown to be downregulated in patient samples, related to a bad diagnosis and treatment, and biologically linked to cancer etiology (Meganck et al., 2018). CircRNA overexpression constructs transferred by adeno-associated virus (AAV) vectors, which do not incorporate into the genome and are presently used in clinical studies, are recently used as a therapeutic solution. On another side, tumorigenic circRNAs, such as the TNBC-specific circAGFG1 and circANKS1B, may be used as new therapeutic targets for TNBC, which currently have few treatment choices and a weak prognosis (Bianchini et al., 2016). To target overexpressed circRNA expression therapeutically, effective approaches have been designed, including degradation mediated by siRNA, shRNA, or altered antisense oligonucleotides (ASOs) complementary to the back-splice junction (Cortés-López and Miura, 2016; Santer et al., 2019). More steady knockout methods, such as CRISPR/Cas genome editing, have also been evaluated. The CRISPR/Cas13 system is a new addition that achieves circRNA silencing by attacking Cas13 to the circRNA's back-splice junction via a specific guide RNA that can differentiatedifferentiate between linear transcripts and circRNAs (Li et al., 2021). Cas13d, a small version of Cas13 (Konermann et al., 2018), in particular, may be packaged into an AAV vector for transmission into primary cells and rodents (Konermann et al., 2018; Zhang, 2021). However, since the side effects of Cas13 expression are currently uncertain, the drawbacks of this system in a clinical sense are not known (Li et al., 2021).

6 HOMOLOGOUS RECOMBINATION DEFICIENCY IN BREAST CANCER GENES

Genomic mutations and instability are the attributions of human cancers that occur due to defective DNA repair mechanisms. One such DNA repair process is homologous recombination (HR), which facilitates the repair of double-strand breaks and

interstrand cross-links (Li and Heyer, 2008). Mutations in BRCA1 and BRCA2 genes are centrally involved in homologous recombination (HR), DNA damage repair, and cell cycle checkpoint regulation (Joosse, 2012). About 5%–10% of breast cancers are associated with inherited mutations in BRCA1 and BRCA2 genes (Institute, 2013). A recent study reported the significance of germline mutations of BRCA1 and BRCA2 by determining their sensitivity to platinum-based chemotherapy and PARP inhibitors (Von Minckwitz, 2014). HRD-mutational signatures are clinically associated with platinum-based chemotherapy in the advanced–stage of breast cancer (Davies et al., 2017).

7 CONCLUSION

Breast cancer is the second mostly reported cancer in women showing a high mortality rate worldwide annually. Early diagnosis and prognosis can control its fatality rate up to some extent. No single biomarker is involved in its diagnosis but a group of multiple diagnostic biomarkers plays a key role in its detection, prognosis, and treatments. Several macromolecules are reported as the significant diagnostic biomarkers including circular RNA, miRNA, DNA, protein, exosomes, and antibodies. Identification of these macromolecules can help in the detection of cancer. DNA methylation and miRNA profiling are the prominent approaches through which breast cancer can be identified.

AUTHOR CONTRIBUTIONS

SA conceived the idea of study. MH, SU, and HA wrote the manuscript. FT helped in data collection. MK and SA proofread the article. All authors approved the manuscript.

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frontiers in Molecular Biosciences

ORIGINAL RESEARCH
published: 23 February 2022
doi: 10.3389/fmolb.2022.823911

Immune Cell Infiltration and Relevant Gene Signatures in the Tumor Microenvironment that Significantly Associates With the Prognosis of Patients With Breast Cancer

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OPEN ACCESS

Edited by:
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Shanghai Jiao Tong University, China

Reviewed by:
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Specialty section:
This article was submitted to Molecular Diagnostics and Therapeutics, a section of the journal Frontiers in Molecular Biosciences

Received: *15 December 2021*
Accepted: *04 February 2022*
Published: *23 February 2022*

Citation:
Xu Q, Yan X, Han Z, Jin X, Jin Y, Sun H, Liang J and Zhang S (2022) Immune Cell Infiltration and Relevant Gene Signatures in the Tumor Microenvironment that Significantly Associates With the Prognosis of Patients With Breast Cancer. Front. Mol. Biosci. 9:823911. doi: 10.3389/fmolb.2022.823911

Breast cancer is the most common malignancy and the leading cause of cancer-related deaths in women. Recent studies have investigated the prognostic value of the tumor microenvironment (TME)-related genes in breast cancer. The purpose of this research is to identify the immune-associated prognostic signature for breast cancer evaluate the probability of their prognostic value and compare the current staging system. In this study, we comprehensively evaluated the infiltration patterns of TME in 1,077 breast cancer patients downloaded from TCGA by applying the ssGSEA method to the transcriptome of these patients. Thus, generated two groups of immune cell infiltration. Based on two groups of low infiltration and high infiltration immune cell groups, 983 common differentially expressed genes were found using the limma algorithm. In addition, studying potential mechanisms, the GSEA method was used to indicate some pathways with remarkable enrichment in two clusters of immune cell infiltration. Finally, the seven immune-associated hub genes with survival as prognostic signatures were identified by using univariate Cox, survival, and LASSO analyses and constructed a TME score. The prognostic value of the TME score was self-validated in the TCGA cohort and further validated in an external independent set from METABRIC and GEO database by time-dependent survival receiver operation. Univariate and multivariate analyses of clinicopathological characteristics indicated that the TME score was an independent prognostic factor. In conclusion, the proposed TME score model should be considered as a prognostic factor, similar to the current TNM stage, and the seven immune-related genes can be a valuable potential biomarker for breast cancer.

Keywords: breast cancer, tumor microenvironment, gene, TME score, prognostic

INTRODUCTION

Breast cancer is one of the major malignancies among females, and mortality remains the second main cause of cancer-related deaths worldwide (Siegel et al., 2019). Currently, similar to other cancers, the diagnosis of breast cancer mainly depends on pathological tests, imaging examinations, and the assessment of tumor markers (McDonald et al., 2016). The TNM staging system is a wildly

used in clinical practice to guide treatment decisions and predict the prognosis of breast cancer patients. For better prognostic value, the current version of the TNM stage has been updated in the past decade, but it has not increased significantly (Nitsche et al., 2011). Recently, increasing attention has been paid to the research on the tumor microenvironment (TME) for its tremendous potential development capacity in the prognosis of patients with breast cancer (Baxevanis et al., 2021).

More studies have shown the role of the TME in cancer progression and therapeutic responses (Jiang et al., 2018). Emerging evidence related to TME revealed that it is crucial to patient outcomes. (Fridman et al., 2012). Successful elimination of tumors through immunotherapy requires activating the immune system. Unfortunately, the depletion or short-lived activation of immune cells and inhibition of microenvironment formation leads to resistance to immunotherapy (Tang et al., 2016). In addition to its effects on immunotherapy, the efficiency of chemotherapy and radiotherapy is also affected through its preexisting properties and induction therapy (Klemm and Joyce, 2015). Immune-associated genes and infiltration of immune cells in TME play a vital role in the properties of tumors, such as proliferation and development (Gonzalez et al., 2018). Therefore, characterizing the immune-associated genes with overall survival may present a prospective reference for breast cancer therapy and prognosis.

In this study, we used computational algorithms based on bulk tumor expression data that systematically profiled the immune cell landscape of the TME in 1,077 breast cancers (Aran et al., 2017). Then, we identified the signature of seven immune-associated hub genes that are related to the prognosis of differentially expressed genes (DEGs). Finally, we constructed a TME score that can be a novel prognostic factor for breast cancer instead of current the TNM stage.

MATERIALS AND METHODS

Collection and Clustering of Breast Cancer Data

We systematically searched for the public breast cancer gene-expression datasets with fully annotated clinical data. We gathered 1,077 patient data on breast cancer genes and retrieve the corresponding clinical information from The Cancer Genome Atlas (TCGA) data portal (Weinstein et al., 2013). All data were analyzed using R (version 3.6.1) and R Bioconductor packages. The gene expression of GSE 103091 was obtained from Gene Expression Omnibus (GEO) database. 1904 samples from the METABRIC cohort were included in this study for further validation (**Supplementary Table S1**). We acquired 28 immune-related cells and types for further analysis (Charoentong et al., 2017). The different TME cell infiltration patterns with tumors were grouped using hierarchical agglomerative clustering (based on Euclidean distance and Ward's linkage). The ssGSEA was performed on the types of immune infiltrating cells, immune-associated functions, and the pathways related to immunization in the expression data of breast cancer using the R package "GSVA". A consensus clustering algorithm was applied to determine the number of clusters in the meta-data set and the Asian Cancer Research Group (ACRG) cohort to assess the stability of the discovered cluster. This procedure was performed using the Consensus Cluster Plus R package and was repeated 1,000 times to ensure the stability of the classification (Monti et al., 2003). According to the results of ssGSEA, low- and high- infiltration immune cell groups were classified in breast cancer samples from TCGA.

Verification of the Effectuality of Immune Clustering

Using the R package "ESTIMATE", gave immune and stromal cells in the TME scores based on the expression levels of specific genes (Yoshihara et al., 2013). ESTIMATE algorithm was used to count the tumor purity of 1,077 breast cancer samples to validate the effectuality of ssGSEA clustering and to create a heatmap and statistical map. Using the R package "ggpubr" generated the vioplots of ESTIMATE score, immune score, stromal score, and tumor purity in the two clusters. A principal component analysis (PCA) was applied using the R package "ggord" to further verify the cluster grouping. In order to investigate the difference among immune cell subtypes, we used hierarchical cluster by ConsensusClusterPackage to count the proportion of 28 immune cells in all breast cancer samples (Charoentong et al., 2017). We also used K-M analysis to validate the difference between two clusters by using the R package "survival".

Identification of Differentially Expressed Genes in Breast Cancer

The patients were grouped into two TME clusters based on immune cell infiltration for identifying the genes associated with TME cell infiltration patterns. The DEGs among these group was deterR package (Ritchie et al., 2015), which implements a Bayesian approach to estimate gene expression changes using moderated t-tests. DEGs among TME subtypes were determined by significance criteria (logFC>1 and $p < 0.01$) as implemented in the R package limma. Gene set enrichment analysis was performed between low- and high- immune cell infiltration clusters of DEGs using R package "ClusterProfiler" (Yu et al., 2012).

Distinction and Conformation of Immune-Associated Gene Prognostic Signature for Breast Cancer

We downloaded clinical information with breast cancer in the TCGA dataset and used univariate Cox analysis to discern the immune-associated gene that was significantly associated with overall survival using the R "survival" package. Then, the LASSO regression analysis was performed to screen the hub genes according to the results in the univariate Cox regression analysis related to survival using the R "glmnet" package. A 1000-round cross-validation for tuning parameter selection was used to prevent overfitting and the partial likelihood deviance met the minimum criteria. Finally, we used the

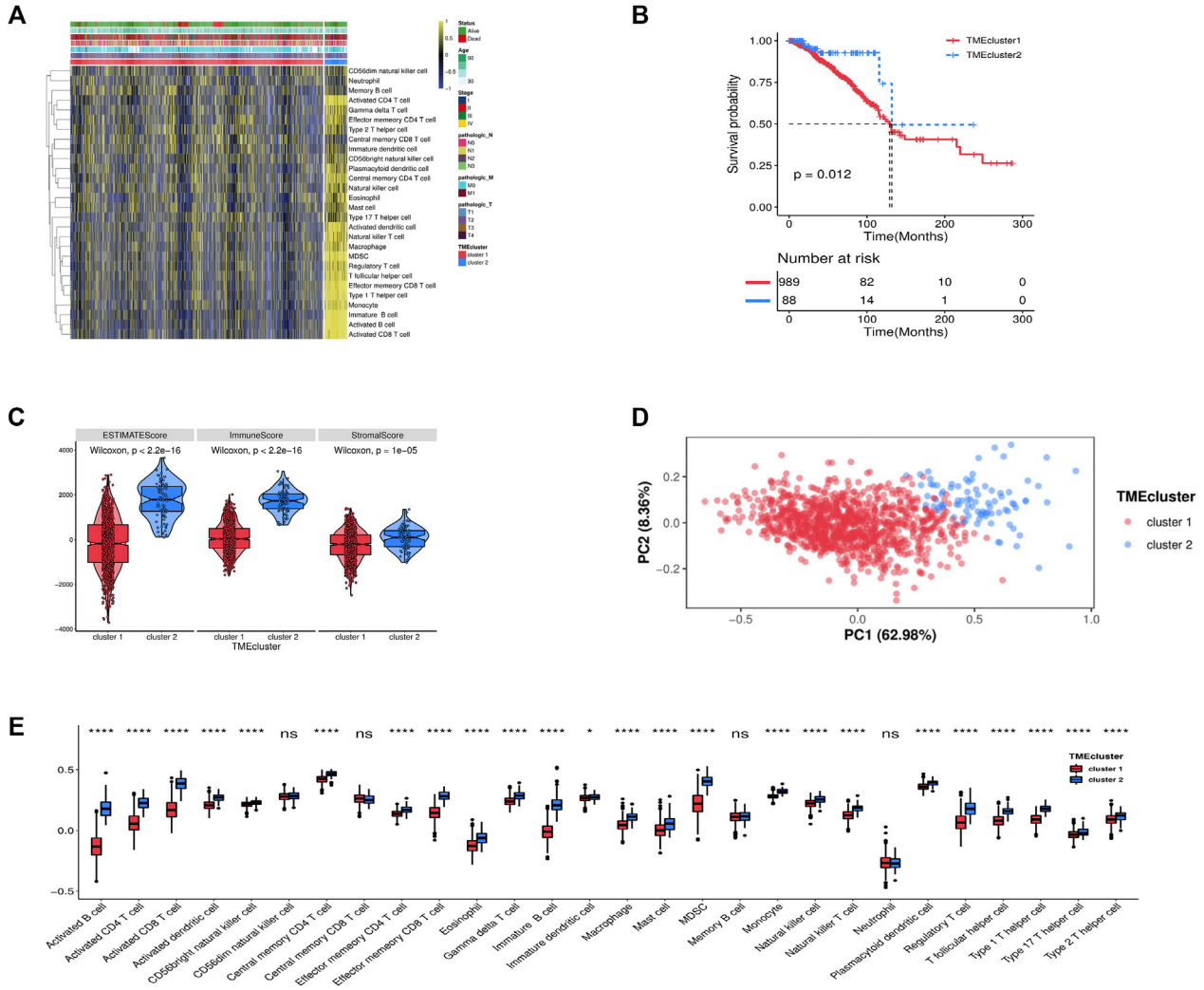

FIGURE 1 | (A) Unsupervised clustering of TME cells for 1,077 patients in the TCGA cohort. **(B)** Kaplan-Meier curves for OS of 1,077 patients in TCGA cohort (log-rank test, $p < 0.001$). **(C)** Expression difference of ESTIMATE score, Immune Score, Stromal Score, and Tumor Purity in two clusters. **(D)** Principal component analysis (PCA) of two TME clusters modification pattern. **(E)** The fraction of TME cells in two clusters. The statistical difference of two TME clusters was compared through the Kruskal-Wallis test. *$p < 0.05$; **, $p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

LASSO regression analysis to generated a prognostic signature of breast cancer via the expression level of immune-related hub genes and their relevant coefficients. The Kaplan-Meier (K-M) curves and time-dependent receiver-operating characteristic (ROC) were applied to assess the clinical prognostic capability of the TME score which was constructed by hub genes using the R packages "survival" and "survminer". Moreover, to assess whether the TME score can indeed be rated as an independent factor of overall survival of breast cancer patients, multivariate and univariate Cox regression analyses were performed with TME score and clinicopathological characteristics as variables using the R package "survival" again.

Statistical Analysis

The normality of the variables was tested using the Shapiro-Wilk normality test (Ghasemi and Zahediasl, 2012). For comparisons of two groups, statistical significance for normally distributed variables was estimated by unpaired Student's t-tests, and non-normally distributed variables were analyzed using the Mann-Whitney *U* test. For comparisons of more than two groups, Kruskal-Wallis tests and one-way analysis of variance were used as nonparametric and parametric methods, respectively (Hazra and Gogtay, 2016). Correlation coefficients were computed using the Spearman and distance correlation analyses. Two-sided Fisher's exact tests were used to analyze the contingency tests. To identify significant genes in the differential gene analysis we applied the Benjamini-Hochberg method to convert the *p* values to false discovery rates. The K-M method was used to generate survival curves for the subgroups in each data set, and the statistical significance of differences was determined using the log-rank test. The hazard ratios for univariate analysis were calculated using a univariate Cox proportional hazards model.

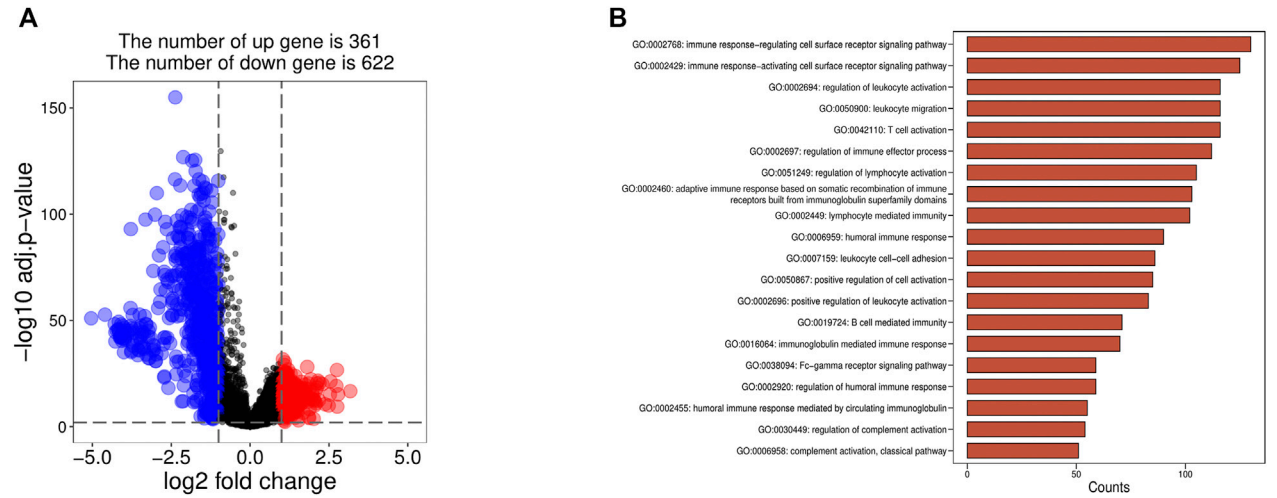

FIGURE 2 | (A) The DEGs visualization was screened by limma. **(B)** GO enrichment analysis of the DEGs.

A multivariate Cox regression model was generated using the heatmap function. All statistical analyses were conducted using the R software. Statistical significance was set at $p < 0.05$ (two-sided).

RESULT

Generation and Validation of Breast Cancer Clustering

We obtained 1,077 breast cancer data from the TCGA. To realize the status of immune cell infiltration, the transcriptome data of breast cancer samples were grouped by the ssGSEA. A sufficient 28 immune-related cells and types in breast cancer samples were obtained. An unsupervised hierarchical clustering algorithm was used to assign the breast cancer samples into two clusters (cluster 1 and cluster 2) based on immune infiltration (**Figure 1A**). Also, we found that the low infiltration of immune cell cluster survival rate was significantly worse than the high infiltration cluster through the K-M curve in breast cancer patients (**Figure 1B**, **Supplementary Table S2**). The ESTIMATE score, stromal score, immune score, and tumor purity were calculated based on the infiltration level of breast cancer using the ESTIMATE algorithm to reflect the availability of the above clustering result. The violin plot has shown that the high infiltration of immune cell cluster (cluster 2) has a higher score than the low infiltration of immune cell cluster (cluster 1) in the stromal score, immune score, and ESTIMATE score (**Figure 1C**). The PCA plot further validated the precision of cluster grouping (**Figure 1D**). **Figure 1E** indicates that the significant differences in immune cell infiltration in the two TME clusters.

Identification of DEGs Between Low and High Infiltration of Immune Cell Clusters

We identified the DEGs between the high infiltration of the immune cell group (cluster 2) and the low infiltration of the immune cell group (cluster 1) by using a significance criterion (logFC>1 and adj. $p < 0.01$). A total of 983 DEGs were preliminarily screened by limma and obtained 361 upregulated and 622 downregulated genes, respectively (**Figure 2A**).

Functional Annotation by GSEA Enrichment Analysis

The GO analysis showed that genes in the high and low immune cell infiltration cluster in the TCGA database were almost related to immune response both in regulating and activating the cell surface receptor signaling pathway, regulation of leukocyte activation, and so on (**Figure 2B**).

Distinction and Evaluation of Seven Immune-Associate Genes Prognostic Signature for Breast Cancer

For further analysis, we used 1,077 breast cancer samples with complete clinical data. Univariate Cox regression analysis and LASSO regression analysis were used to identify seven immune-associated genes, including *SEC14L2, IGHD, IGHA1, CHAD, PCSK6, BIRC3*, and *CCDC74B*, which were most significantly associated with overall survival (**Figures 3A,B**). The details of immune-related genes are comprehensively displayed in the circus plot (**Figure 3C**). We further studied the prognosis value of each immune-related gene for breast cancer patients and demonstrated its correlation in immune cell infiltration (**Figures 3D,E**).

Then, we assigned patients into high TME score and low TME score groups using the cutoff value (cut off -1.904) obtained with the Survminer package. Kaplan-Meier (K-M) curves and log-rank test were revealed that the low TME score group had a significantly better survival than the high TME score group ($p < 0.001$), showing that the TME score has an effective prognostic value (**Figure 4A,B**, **Supplementary Table S2**). Then we also test the prognosis value of seven immune-associated genes in METABRIC cohort. It showed

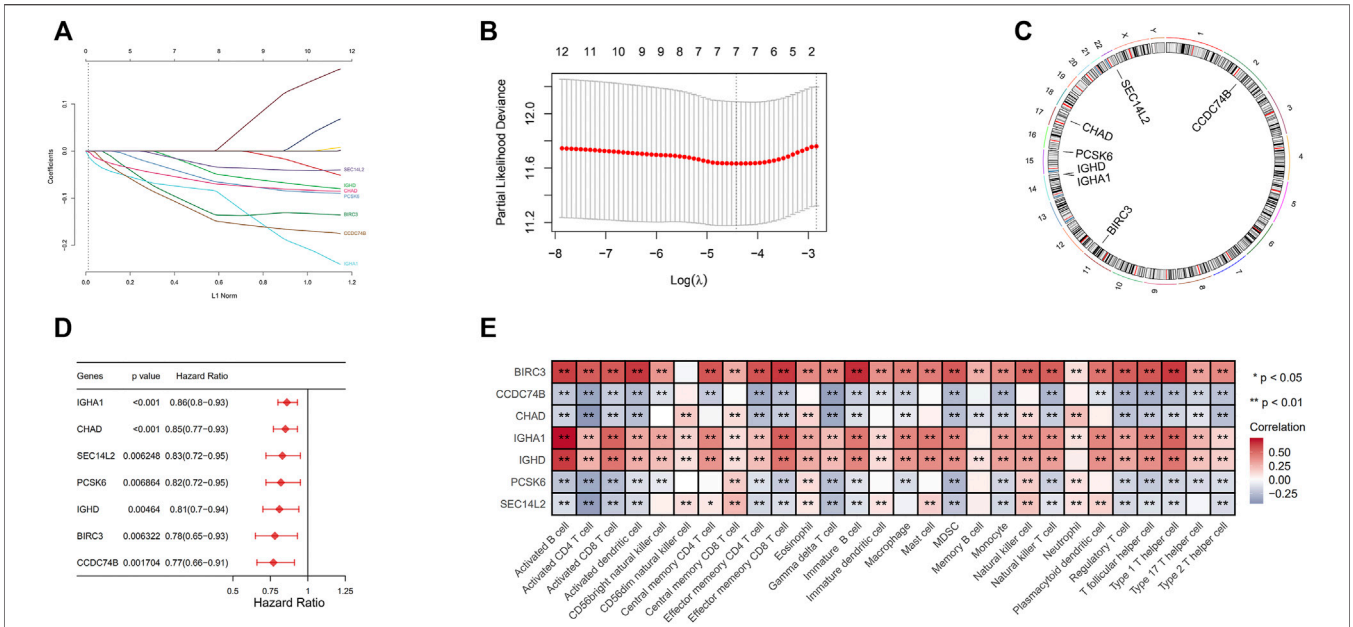

FIGURE 3 | (A) The optimal penalty parameter values were confirmed by 1,000 round cross-validation. **(B)** Seven hub genes related to prognosis were analyzed by LASSO Cox analysis. **(C)** Each of seven genes on chromosomes was shown in Circos plots. **(D)** The contribution made by each of the seven genes to survival differences. **(E)** Correlation matrix of immune cell infiltration and the expression levels of seven immune-associated genes.

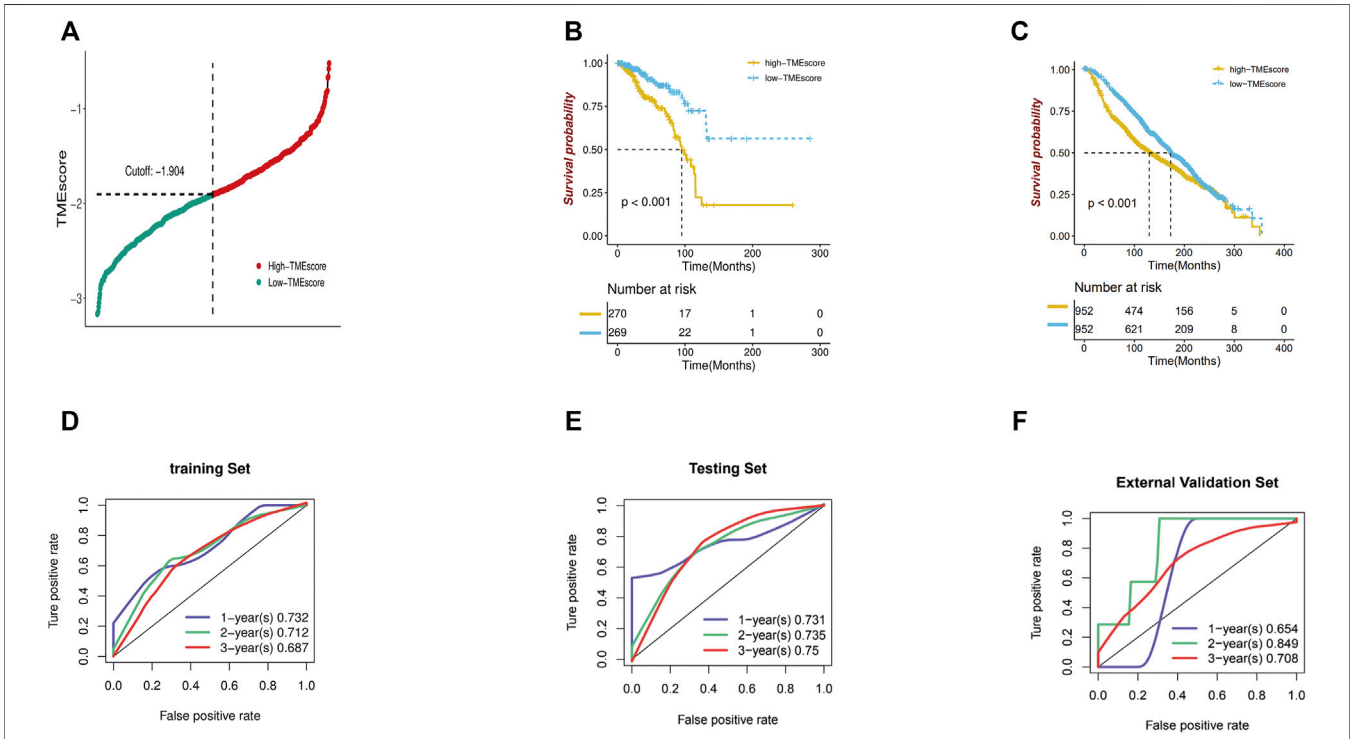

FIGURE 4 | (A–C) The TME score and Kaplan-Meier curve analysis of seven immune-associated gene signature in TCGA-BRCA and METABRIC cohort. **(D–F)** ROC curves measuring the predictive values of the TME score at 1, 2, and 3 years in overall, training and external validation set, respectively.

TABLE 1 | Univariate and multivariate analyses of clinicopathological characteristics and TMEscore with overall survival in TCGA BRCA cohort.

Characteristics	Univariate analysis HR (95% CI)	*p*value	Multivariate analysis HR (95% CI)	Pvalue
TMEscore	2.875 (2.008–4.117)	<0.001	2.674 (1.76–4.061)	<0.001
Age	1.032 (1.02–1.045)	<0.001	1.029 (1.015–1.044)	<0.001
Stage				
Stage II	1.59 (0.92–2.749)	0.097	1.29 (0.525–3.166)	0.579
Stage III	3.033 (1.71–5.38)	<0.001	2.032 (0.578–7.144)	0.269
Stage IV	13.035 (6.426–26.438)	<0.001	5.306 (1.333–21.123)	0.018
pathologic_T				
T2	1.298 (0.863–1.953)	0.211	0.957 (0.497–1.842)	0.896
T3	1.576 (0.935–2.655)	0.087	0.895 (0.378–2.119)	0.801
T4	3.976 (2.142–7.378)	<0.001	1.283 (0.483–3.409)	0.617
pathologic_M	4.869 (2.906–8.157)	<0.001		
pathologic_N				
N1	1.851 (1.252–2.735)	0.002	1.42 (0.841–2.398)	0.19
N2	2.743 (1.641–4.585)	<0.001	1.812 (0.712–4.609)	0.212
N3	4.106 (2.27–7.428)	<0.001	1.778 (0.717–4.41)	0.214

HR, hazard ratio; CI, confidential interval.

that low TME score group also has better survival than high TME score group (**Figure 4C**).

Assessment of Tumor Microenvironment Score as Independent Prognostic Factor in TCGA and GSE103091

The multivariate and univariate Cox regression analyses are applied to test whether the TME score calculated by the seven immune-associated hub genes was a latent independent prognostic factor. The Univariate and multivariate Cox regression analyses showed that TME score can be an independent prognostic factors ($p < 0.001$) (**Table 1**).

In addition, the TME score stably maintained a powerful and independent factor in the training set, testing set, and external validation sets. Time-dependent ROC was applied to evaluate the precision of predicting overall survival of breast cancer at 1, 2 and 3 years between the high and low TME scores. The area under the ROC (AUC) values at 1,2,3 years were 0.732,0.712, and 0.687, respectively, in the training set and 0.731,0.735, and 0.75, respectively, in the internal testing. To validate whether our prognostic classifier had similar predictive ability in different populations, we applied it to the external validation set (GEO103091), and the result is 0.654, 0.849, and 0.708 at 1, 2, and 3 years, respectively (**Figures 4D–F**). The GO analysis of the low TME score group has showed that these were associated with B cell activation, cell chemotaxis, cell junction organization, cell matrix adhesion, cell substrate adhesion and more (**Supplementary Figure S1**). The KEGG analysis of the low TME score group has indicated that they were associated with the JAK-STAT signaling pathway, cytokine receptor interaction, leukocyte *trans*-endothelial migration, and so on (**Supplementary Figure S2**).

DISCUSSION

Breast cancer is the most frequent and deadly malignant tumor among women around the worldwide, because of its complicated TME, it is a highly heterogeneous disease (Sousa et al., 2019). The high heterogeneity of breast cancer exists in the molecular level of tumor cells, as well as in the TME (Baker et al., 2018). In addition, breast cancer tissue is not just composed of cancer cells, also mixed with several types of normal cells, such as immune cells, stromal cells, and fibroblasts (Bai et al., 2019). The relationship between TME and the properties of *BRCA*, such as tumor progression, invasion, and metastasis has been widely recognized (Bussard et al., 2016). Thus, we identified and validated the prognosis value of seven immune-associated genes with the survival of breast cancer datasets obtained from TCGA.

In this study, unsupervised hierarchical clustering algorithm was applied to classified the samples into two clusters based on the enrichment of 28 immune cell types. There were significant differences between low- and high- infiltration of immune cell clusters in the immune score, stromal score, ESTIMATE score, and tumor purity. In addition, the K-M analysis revealed that the breast cancer patients in the high immune cell infiltration cluster had a higher survival rate and that the survival rate of the two clusters was significantly different. We also discovered seven innovative immune-related genes based on the TCGA-BRCA cohort, which were successfully validated in an external independent set from METABRIC and GEO cohort. The overall survival time of the low-TME score group was significantly better than that of the high-TME score group. Univariate and multivariate Cox regression analyses showed that the seven immune-associated hub genes were an independent prognostic factors in both TCGA and GES 103091 datasets. Moreover, the AUC confirmed that the seven immune-related genes were comparable and superior to the TNM stage in predicting the overall survival of breast cancer patients. To further analyze the relationship between the TME score group and TNM stage. Therefore, these results suggest an excellent prediction capability for the seven immune-associated hub genes.

Seven immune-related hub genes, including *SEC14L2, IGHD, IGHA1, CHAD, PCSK6, BIRC3*, and *CCDC74B* were studied. Proprotein convertase subtilisin/Kexin type 6 (PCSK6) is a

proteinase that regulates the proteolytic activity of various precursor proteins and protein maturation. A previous study revealed that PCSK6 significantly enhanced cell motility, migration, and invasion abilities when they overexpressed in MDA-MB-231 breast cancer cells *in vitro* (Lapierre et al., 2007). In addition, blocking PCSK6 expression in breast cancer MA-MB-231 cells inhibited their proliferation, invasion and migration abilities (Wang et al., 2015). In addition, PCSK6 was also reduced cell cycle arrest and prevented apoptosis of MDA-MB-231 cells and increased the expression level of the phosphorylated forms of ERK1/2 and WNT3A. Meanwhile, CCDC74B is a k-fiber crosslinker required for chromosomal alignment, thus by promoting the k-fiber stability and maintaining the spindle integrity to ensure proper chromosome alignment and cell division (Zhou et al., 2019). SEC14L2/TAP (tocopherol-associated protein) is a tocopherol-binding protein that regulates transcription and cholesterol metabolism (Porter, 2003). It is highly expressed in the breast, prostate, liver, and brain, the contrast was observed in many human tissues (Zimmer et al., 2000). A previous study demonstrated that TAP was downregulated in breast cancer; therefore, TAP/SEC14L2 may function as a tumor suppressor in breast tumors (Wang et al., 2009). BIRC3 (cellular IAP2) is a member of the human IAP family (Liang et al., 2020). It is overexpressed in malignant breast cancer compared to primary breast cancer, and is also linked to resistance to anti-cancer agents (Frazzi, 2021). Chondroadherin (CHAD) is a cartilage matrix protein thought to mediate adhesin in isolated chondrocytes (Camper et al., 1997). Low levels of CHAD have been associated with poor survival in hepatocellular carcinoma (Deng et al., 2017). IgA are produced in the airways and gastrointestinal tract, as well as in the lactating breast. IGHA1 and IGHA2 mRNA levels are highly correlated and are associated with improved prognosis with a higher immune activity in breast cancer (Larsson et al., 2020). IgD is a membrane-bound B cell receptor, and the B cell express IgD before the class switch. These results confirmed that the seven TME-associated immune genes affect the prognosis of patients with cancer.

We then further investigated the correlation between TME score and clinical characteristics in patients with breast cancer using the multivariate Cox and univariate Cox regression analyses. According to the result, the TME score showed good potential as an independent prognostic factor in patients with breast cancer ($p < 0.001$). Moreover, some studies have clarified the probability of TME score as a prognostic factor in several cancers. It is demonstrated its predictive value for immune checkpoint blockade (Zeng et al., 2019).

The limitations of our study are as followed. All the data and results were through a drying test and further research and confirmation is needed to reduce the bias from clinical practice, such as actual animal experiments.

In conclusion, we developed and validated the TME score which could be independent prognostic signatures for breast cancer based on the seven immune-related gene signatures. Our study may guide the prediction of prognosis and survival in patients with breast cancer and may provide potential targets for immunotherapy.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

S-Z proposed the topic. Q-X, X-Y, Data curation, Formal analysis, Writing—original draft. Q-X, X-Y, Z-H, X-J, Y-J, H-S, and J-L conducted the calculation and prepared all the figures in R. All authors discussed the results and approved the final manuscript.

FUNDING

This work was supported by the National Nature Science Funds of China (Nos.81560478).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2022.823911/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
published: 28 March 2022
doi: 10.3389/fmolb.2022.781111

Interrelated Oncogenic Viruses and Breast Cancer

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Breast Cancer is a multifactorial disease and recent evidence that viruses have a greater role in its aetiology and pathophysiology than previously hypothesized, has garnered a lot of attention in the past couple of years. After the role of Mouse Mammary Tumour Virus (MMTV) in the oncogenesis of breast cancer has been proved in mice, search for similar viruses found quite a plausible relation of Human Papilloma Virus (HPV), Epstein–Barr virus (EBV), and Bovine Leukaemia Virus (BLV) with breast cancer. However, despite practical efforts to provide some clarity in this issue, the evidence that viruses cause breast cancer still remains inconclusive. Therefore, this article aims to clarify some ambiguity and elucidate the correlation of breast cancer and those particular viruses which are found to bring about the development of tumorigenesis by a previous infection or by their own oncogenic ability to manipulate the molecular mechanisms and bypass the immune system of the human body. Although many studies have reported, both, the individual and co-existing presence of HPV, EBV, MMTV, and BLV in patient sample tissues, particularly in Western women, and proposed oncogenic mechanisms, majority of the collective survey of literature fails to provide a delineated and strong conclusive evidence that viruses do, in fact, cause breast cancer. Measures to prevent these viral infections may curb breast cancer cases, especially in the West. More studies are needed to provide a definite conclusion.

Keywords: breast cancer, human papilloma virus, Epstein–Barr virus, mouse mammary tumor virus, bovine leukemia virus

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Specialty section:
This article was submitted to Molecular Diagnostics and Therapeutics, a section of the journal Frontiers in Molecular Biosciences

Received: *22 September 2021*
Accepted: *15 March 2022*
Published: *28 March 2022*

Citation:
Afzal S, Fiaz K, Noor A, Sindhu AS, Hanif A, Bibi A, Asad M, Nawaz S, Zafar S, Ayub S, Hasnain SB and Shahid M (2022) Interrelated Oncogenic Viruses and Breast Cancer. Front. Mol. Biosci. 9:781111. doi: 10.3389/fmolb.2022.781111

INTRODUCTION

Breast cancer is a chronic disease, primarily owing to its metastatic characteristics, due to which it can relocate to other parts of the body such as bone, liver, brain and lungs. With 2.3 million women diagnosed, and 685,000 worldwide deaths in the year 2020 alone, breast cancer continues to retain its place as the most prevalent cancer in the world (Breast Cancer, 2021). The incidence of breast cancer in Pakistani women is increasing at an alarming rate of 19.33% every year, owing to the fact that one in every nine woman suffers from it (Baloch et al., 2012). Due to this, the ratio of breast cancer in Pakistani women is the most in the whole of Asia (Baloch et al., 2012).

Breast cancer is becoming increasingly common in women, as a 150 times higher number of cases are reported in women as compared to men (Lawson, 2009). However, it also occurs as a rare malignancy in adult older males, with approximately 1% reported cases (Siegel et al., 2016). The liability of occurrence and mortality of breast cancer is four to five-fold higher in Western women as compared to Eastern, particularly, Asian women (Ghocheh et al., 2015). Moreover, high mortality

rates are observed in less developed countries as compared to more developed countries, which have a high prevalence (Ghocheh et al., 2015).

A large number of internal and external factors bring about the development of breast cancer. Some significant external factors that run the risk of inducing breast cancer include: genetic susceptibility (e.g., mutations in BRCA1/2 and other genes), obesity, a familial history of breast cancer, unhealthy behavioural choices, hormonal contraception and treatment after menopause (Lawson, 2009). Besides genetics, some other external factors that can contribute to breast cancer initiation are variation of food consumption among populations, and difference in fertility pattern (Lawson, 2009).

However, among such factors, despite that around 16% of all human cancers are caused by biological role carcinogens, the role of oncogenic viruses is less obvious (de Martel et al., 2012). Since a couple of decades, the evidence that viruses may play an influential role in the pathogenesis of breast cancer is becoming increasingly clear. Among such oncogenic viruses, the role of Human Papilloma Viruses (HPVs), Epstein–Barr virus (EBV-also known as human herpes virus type 4), Mouse Mammary Tumour Virus (MMTV) and Bovine Leukemia Virus (BLV) particularly stand out. In recent literature, reported information regarding the presence of viruses and their manipulation of molecular mechanisms for tumorigenesis is somewhat ambiguous and contradictory, and remains yet to be annotated (Lawson et al., 2018). Therefore, the aim of this article is to shed some light on the correlation of the presence of HPV, EBV, MMTV and BLV, their infections, and their oncogenic ability by which they exploit the human molecular pathways and the immune system to bring about the pathogenesis of breast cancer.

HUMAN PAPILLOMA VIRUS AND BREAST CANCER

Human Papilloma Virus (HPV) is small in size, circular and contains double stranded DNA as its nucleic acid. About 200 different species of HPVs are classified into two categories: mucosal and cutaneous (Münger et al., 2004). Infection due to HPV is majorly transmitted through sexual activities or by skin-to-skin contact (**Figure 1A**). These are mostly short lived

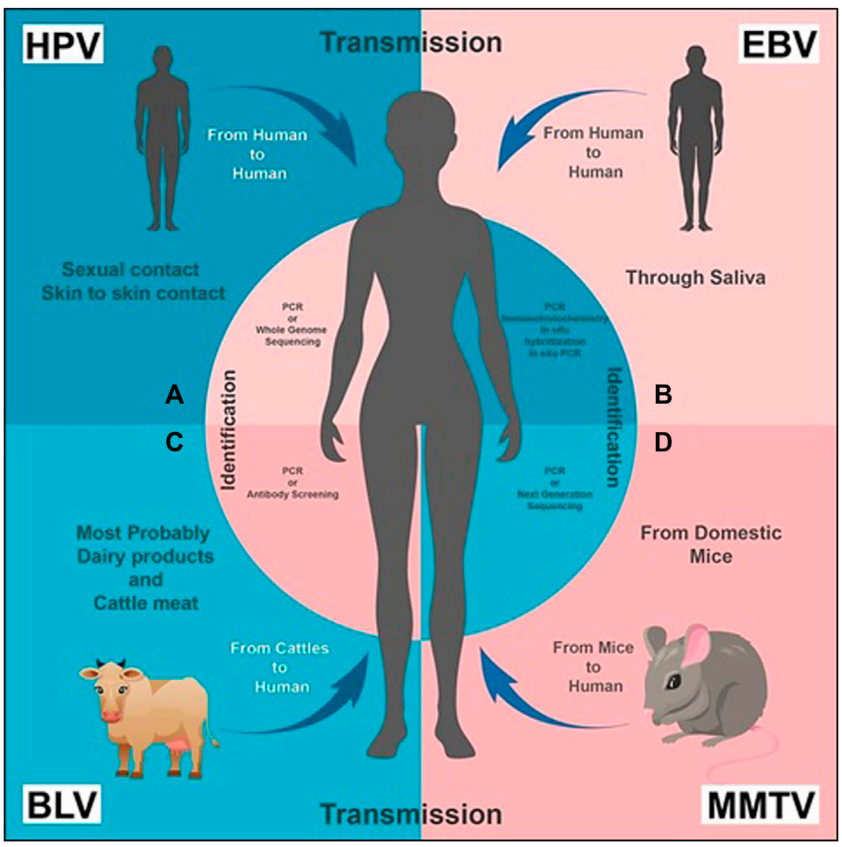

FIGURE 1 | Transmission and Identification of the viruses, respectively: **(A)** HPV transmission takes place from human to human through sexual and/or skin to skin contact. It can be diagnosed by PCR (recommended) or Whole Genome Sequencing. **(B)** EBV is transmitted through saliva from human to human. Its presence can be identified by PCR, immunohistochemistry, *in situ* hybridization and *in situ* PCR. **(C)** BLV transmission primarily takes place from bovine or cattle (usually domestic animals) to humans; dairy and cattle meat can also be a source of transmission. PCR or antibody screening of a human blood sample is carried out for its diagnosis. **(D)** MMTV is transmitted from mice to human. Its presence is identified by PCR or NGS.

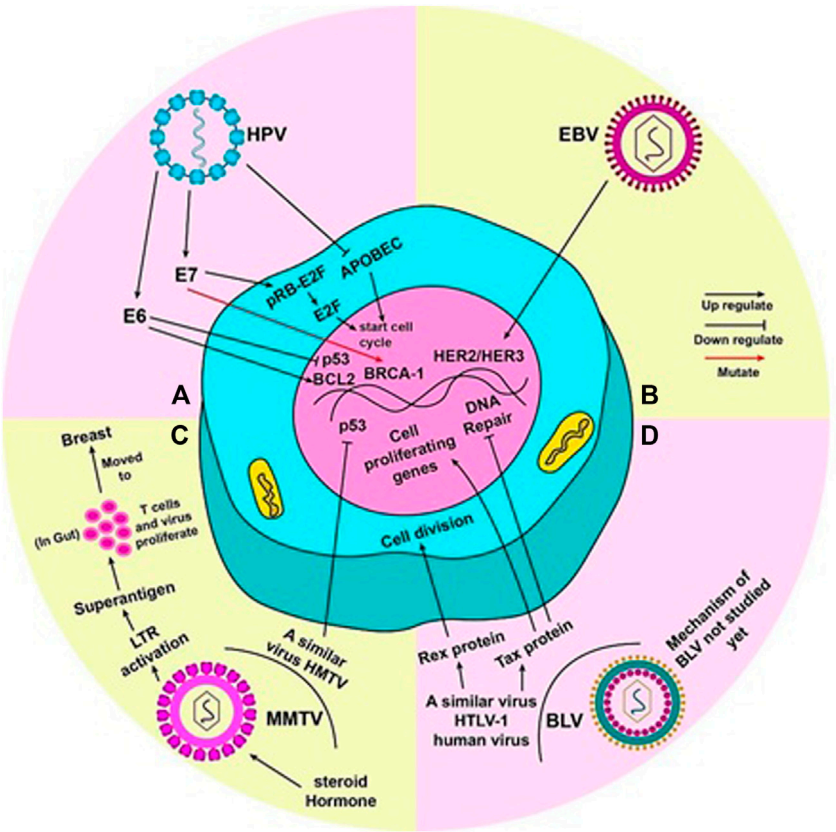

FIGURE 2 | Mechanism by which each virus is said to cause breast cancer: **(A)** When mutated, oncoproteins (E6 and E7) of HPV virus activate pro-tumor genes. E6 activates BCL2 and inhibits p53, thus, promoting cell division. The pRB-E2f complex inhibits cell cycle; E7 detaches E2f from pRB and E2F alone commences the cell cycle. Mutation of BRCA-1 gene by E7 can also lead to tumor development. HPV also inhibits APOBEC (a cell cycle inhibitory molecule) to start cell cycle and cause genome instability. **(B)** EBV can activate HER2/HER3 gene(s) to render cell towards uncontrolled division. **(C)** Steroid hormones lead to efficient replication and increased expression of MMTV genome. An LTR in the MMTV genome codes for superantigens which activate T lymphocytes. MMTV replicates rapidly in these T cells in the gut, during which it can be carried to the breast. HMTV, a virus similar to MMTV, can inhibit p53 to induce to cell cycle. **(D)** The true mechanism by which BLV causes cancer in humans has not been yet studied. However, HTLV-1, a virus similar to BLV present in humans, can lead to uncontrolled cell division by the production of Rex and Tax proteins. Tax protein accentuates this by activating cell proliferating genes and halting DNA repair mechanism of the cell.

and, within 2 years of infection, 90% of men and women naturally clear the viral load (Winer et al., 2003).

There is evidence that high risk HPV 16 and 18 are involved in cervical carcinoma and other cancers related to genital sites (Shukla et al., 2009). Most of HPV strains are causative agents of cervical cancer, but "high risk" HPVs have been reported for breast cancer, as well (Rassi et al., 2009). HPV-associated cervical infections can precede the development of same type HPV-positive breast cancer in the same patient (Lawson et al., 2016) As a result, HPV viral load is higher in cervical cancer as compared to that in breast cancer (Khan et al., 2008). Due to this, identification of HPV in breast tumours is quite difficult, but it can be done either by whole-genome sequencing, or by PCR (**Figure 1A**). High-risk HPVs have also been identified in the SK-BR-3 breast cancer cell line (Yan et al., 2016). In a study, whole-genome sequencing was used to identify high-risk HPVs in invasive breast cancers (Lawson et al., 2015).

Duct epithelia are the rising ground in most breast cancers; similarly, HPV is reported to enter mammary ducts through the nipple areola complex, which is continuously exposed to the external environment (Polyak, 2007). The cell cycle control enzyme, APOBEC, is altered by viral infection, which causes genomic instability and leads to breast cancer (Ohba et al., 2014). Some studies also report that the early genes of oncoproteins E6 and E7 of HPV 16 inactivate the two major tumour suppressor proteins of the human body, Rb and p53, by interacting with them (Dyson et al., 1989; Werness et al., 1990). This is reported to commence continuous division of mammary epithelial cells in *in vitro* experiments, and in doing so, immortalize them, leading to breast cancer (Wazer et al., 1995; Liu et al., 1999). According to reports of familial breast cancer cases, these oncoproteins are notorious for inducing mutations in *BRCA1* gene, due to the functional interaction that exists between E7 and the *BRCA1* gene (Rassi et al., 2009). E7 of high risk HPV 16 dissociates the pRB-E2F complex by binding to pRB (Zhang et al., 2005). Lower p53 and higher BCL2 level is a hallmark of breast carcinogenic tumours. E6 deregulates expression of p53 and

BCL2 by inactivating p53 and releasing repression of BCL2 (**Figure 2A**) (Sidransky and Hollstein, 1996).

In addition, a research study of the Indian population also observed the role of "high risk" HPV 16 in promoting breast cancer at genetic and epigenetic levels (Islam et al., 2017). They found that frequent integration of HPV into host's genome is seen through disruption of hinge region (E28) of E2 gene, which gradually increases at different stages. Even lower viral loads are associated with cancer induction. Moreover, they confirmed HPV occurrence by sequencing and analysing LCR, E6 and E7 regions of HPV 16 genome. Variation analysis of LCR revealed one common variant (7521G>A) that overlaps with the binding of transcriptional repressor, YY1, regulating the expression of E6 and E7, and four novel variants (7628A>T, 7800A>G, 7837A>G and 7839A>C). The variant of E6 (350T>G) has been shown to be associated with HPV pathogenicity, while the two novel variants (519G>A) and (395G>T), destabilize the E6 mRNA and protein stability. The integration of HPV 16 in breast cancer is also linked to hyper methylation of promoter and enhancer regions of P97. Furthermore, expression analysis of E6 and E7 of HPV 16 showed two spliced transcripts (E61* and E6II*) of E6 and three spliced transcripts (E6*I/E7, E6*II/E7, E6E7) of E6/E7, along with two novel fusion transcripts R6E7*I and E6E7*II in samples of breast cancer hypothesized to be associated with HPV (Islam et al., 2017).

In concordance with results of Ohba et al. (2014), another study suggested that HPV also influences genomic instability by interfering with the cell cycle control enzyme APOBEC, ultimately leading to breast cancer. They also observed that HPV initiates cancer and then disappears from tumour cells by the time cancer is clinically detected, therefore, suggesting a "hit and run" hypothesis for its initial mode of action (Balci et al., 2019).

Reports regarding the causal role of HPV and breast cancer seem to be contradictory in nature. Some studies report the presence of HPV in breast tumour samples either before or during the confirmation of the disease. For example, a study conducted in Iranian females with breast cancer discovered that 25.9% of tumour samples were positive for HPV DNA, and among these, 53.34% were of HPV 16 and 18 (Sigaroodi et al., 2012). Similarly, Salman et al. (2017) reported selective presence of twelve high risk HPVs in 42% of 110 female breast tumour samples, and confirmed viral activity majorly in invasive carcinomas. However, some studies report no or very low expression of HPV in samples associated with breast cancer. For instance, a study executed on Danish women with breast cancer, who were previously affected with cervical dysplasia, found only 1.55% prevalence of HPV in the assays they used (an in-house semi-Q PCR assay and SPF_{10} PCR-DEIA-$LiPA_{25}$) and no particular difference regarding between samples of patients and controls (Bønløkke et al., 2018). Furthermore, from a samples size of 76 breast carcinomas and two benign tumour samples in a research study Spain, no evidence of HPV genome was found by Vernet-Tomas et al. (2015).

Although breast cancer cannot be prevented, one can and must observe some strategies to minimize its risk. Therefore, it's recommended for women to regularly perform self and screening exams, as well as getting frequent mammograms with advancing age (Purdie, 2019). The best way to prevent an HPV infection is to get vaccinated against it. FDA approved drugs for HPV are classified as: human papillomavirus bivalent vaccine (Cervarix); human papillomavirus Quadrivalent vaccine (Gardasil); human papillomavirus 9 violent vaccine (Gardasil 9) (Purdie, 2019). The shots of vaccination differ in Gardasil being specific for ages 9–14, while Gardasil 9 is for people aged 15–26 (Purdie, 2019). According to a survey study, Gardasil 9 protects against nine types of HPV infection, which hold prime importance against genital warts, while the other seven types are fruitful against other cancers caused by HPV, including breast cancer (HPV and Cancer National Cancer Institute, 2021).

Moreover, various other important aspects to treat HPV-related breast cancer are also present. Focusing on molecular targets which facilitate gene therapy is one such way; for example, a recent study showed that knockdown of HPV 18's E6 and E7 oncogene results in inhibition of cancer progression (Yan et al., 2016). Similarly, finding alternative ways of breast cancer detection when target receptors are absent in patients is another useful strategy. For instance, a study reported 15% positivity of HPV in triple negative breast cancer (an aggressive form of breast with poor prognosis), which lacks receptors for ER and PR genes and shows an inability to amplify expression of *HER2* gene (Abramson et al., 2015; Horakova et al., 2018).

EPSTEIN–BARR VIRUS AND BREAST CANCER

Epstein-Barr virus (EBV), a gamma-Herpes virus which belongs to the Herpesviridae family, has a linear double-stranded DNA genome that encodes more than 85 genes. It is commonly found in approximately 95% of the total population. Approximately, 200,000 malignancies all over the world are annually associated with EBV. Its infection is common worldwide, but usually appears later among Western individuals in comparison to the developing communities (Speck and Longnecker, 2000). The most common route of transmission of the Epstein–Barr virus is saliva (**Figure 1B**). Primary infection of EBV causing a mild disease is more frequently reported in childhood. It appears asymptomatic in 20%–80% individuals aged two or three (Bolis et al., 2016; Prabhu and Wilson, 2016). When exposed to EBV, approximately 30%–70% of healthy young individuals can develop infectious mononucleosis (Bolis et al., 2016).

In addition to mononucleosis, EBV is reported as the causative agent of lymphoma, Langerhans cell histiocytosis, and nasopharyngeal cancer (Kang and Kieff, 2015). It is also responsible for oncogenesis in humans, and causes a latent infection that persists lifelong in most afflicted people (Mazouni et al., 2015). A restricted expression of viral proteins (latency III: six nuclear proteins EBNA-1, EBNA-2, EBNA-3A-C, EBNA-LP, and three latent membrane proteins (LMP-1, LMP-2A, and LMP-2B)) characterize its latent infection (Kang and Kieff, 2015). Along with lymphomas, EBV is associated with several other cancers, especially breast cancer, in which it is

reported to induce tumour formation only by cell-to-cell contact (Speck and Longnecker, 2000). Techniques like immunohistochemistry, *in situ* hybridization, standard liquid PCR, and *in situ* PCR have been used to identify EBV genes in breast cancer in various countries (**Figure 1B**) (Hu et al., 2016). Similar methods found out the expression of EBV in some benign breast tissues, and its gene sequences have been identified even before the development of EBV-positive breast cancer. The prior and later specimens were from the same patients (Hu et al., 2016).

The most likely mechanism proposed by which EBV can form tumours is the one in which it activates the *HER2/HER3* signalling cascades, resulting in infection predisposition in breast epithelial cells, leading to the dreaded malignant transformation stage (**Figure 2B**). *HER2* and *HER3* are oncogenes that are known to be involved in the development of human breast cancer with a poor prognosis. CD21 receptors are also involved in transforming primary mammary epithelial cells to malignant cells by EBV infection and are no longer needed by the cells after onset of malignancy (Hu et al., 2016).

Due to the low reported frequency of BRCA1 and BRCA2 genes in Sudanese women with breast cancer, the methylation status of six tumour suppressor genes (*BRCA1*, *BRCA2*, *p16*, *p14*, *MGM2*, and *hMLH*) was observed by Liu et al. (2012). The reported methylation frequencies of the genes were: 84% of *BRCA1*, 84% of *BRCA2*, 15% of *p16*, 81% of *p14*, 12% of *MGM2*, and 18% of *hMLH*. A potent effect of the EBV virus on the methylation machinery is suggested by the high frequency of epigenetic silencing in *BRCA1*, *BRCA2*, and *p14*; this is an oncogenic mechanism delineated in other cancers except in breast cancer (Ryan et al., 2010; Liu et al., 2012). The methylation of *p14* can be a potential mechanism by which the tumour genome evades control of p53 and other related tumour suppressors, thereby, annulling the role of gene mutations. Esteller et al. (2000) first proposed this mechanism in colorectal cancer, and its basis was the over-representation of p14ARF hyper-methylation in tumours with wild-type p53, in comparison to tumours harbouring p53 mutations. Tumour type greatly influences the methylation profiles of tumour suppressor genes, and each tumour is designated with a distinct "DNA hypermethylome" (Estellar, 2005).

Lin et al. (2007) infected MCF7-A and BT474-A cells with EBV, and increased anchorage-independent growth was observed in cells on soft agar. This increase in cell mass was found to be associated with hyper-activation and expression of *HER2/HER3* signalling cascades, as supported by the findings that the treatment of phosphatidylinositol 3-kinase inhibitor, *HER2* antibody trastuzumab (Herceptin), or MEK inhibitor completely ceased the oncogenic capacity. The expression of EBV latency genes *EBER1*, *BARF0*, and *EBNA* was determined in breast cancer cells infected with EBV. However, it has been detected that *BARF0* alone was enough to promote tumorigenic activity in MCF7 and BT474 cells by up-regulating the expression of *HER2/HER3*, utilizing the techniques of small interfering RNA knockdown and overexpression. Consequently, a significant rise in the HER2 protein level was also reported. Yarden and Sliwkowski (2001) stated that HER2 lacks an activating ligand; therefore, it forms heterodimers with HER1, HER3, or HER4 to transfer signals downstream upon ligand binding. The expression status of HER1, HER3, and HER4 was also examined, and it was found that EBV-infected cells showed significantly increased expressions of HER3 at both transcription and translation levels, whereas, the expression of HER1 and HER4 was unaffected. A dramatic increase of tyrosine phosphorylation of HER2 coupled with increased binding of p85 (PI3K subunit) to HER2/HER3 complex was also reported. All these results suggest that EBV infection enhances both the expression and functional activity of HER2/HER3. Collectively, it was demonstrated that EBV-encoded *BARF0* promotes the oncogenic capacity of breast cancer cells by activating the HER2/HER3 signalling cascades.

Since the 1990's, studies have reported the presence of EBV in human B lymphocytes, by which the virus has an easier access to breast epithelial cells for infection (Magrath and Bhatia, 1999). This might make it possible for the virus to commence oncogenesis in those breast epithelial cells (Richardson et al., 2015). However, contradictory results have been gained in regard to this hypothesis. For example, one of the first studies conducted in London centring on the presence of EBV in breast tumour samples showed a positive EBV result (Labrecque et al., 1995). Another study conducted in France reported the presence of EBV in 33.2% breast cancer tumours, which tended to be found in more aggressive cancer types (Mazouni et al., 2011). A very conflicting meta-analysis study reported that the highest prevalence of EBV-associated breast cancer was majorly present in Asia (35.25%), while the least was in the United States (18.27%) (Hou et al., 2012). Some studies report otherwise; Perrigoue et al., (2005) reported no contribution of EBV to breast cancer in their study conducted in America; Herrmann and Niedobitek (2002) found a negative association of EBV with breast cancer; in Mexico, Morales-Sanchez et al. (2013) took 86 tumour tissue samples and 65 tissues adjacent to the site of tumour and checked for the presence of EBV and MMTV to report that conventional PCR results showed no presence of either genome in any of the samples, while a more sensitive test with nested PCR revealed only four tumour samples to be positive for EBV.

Herpes viruses (a family of DNA viruses) predominantly express viral miRNAs (Cullen, 2009). Several viral mRNAs are targeted by viral miRNAs, for example, EBV encodes miR-BART2 that inhibits BALF5 (an EBV DNA polymerase) (Pfeffer et al., 2004). As described in several studies, the use of CRISPR/Cas9 technology interferes with the maintenance/replication of EBV, or edits its genome. CRISPR/Cas9 was used by Yuen et al. (2015) to delete a 558 bp sequence in the promoter region of the BamHIA rightward transcript via NHEJ, in virus-infected cells by direct chopping of the EBV genome. A transgene was introduced by HDR upon co-delivery of a template sequence at the same target site (Yuen et al., 2015). EBV-negative Akata cells can be successfully manipulated by the introduction of recombinant viruses (van Diemen et al., 2016). In addition to the application of CRISPR/Cas9 for the direct production of EBV variants, the use of anti-EBV gRNAs to hamper latent EBV infection has been described by two reports (Wang and Quake, 2014; van Diemen et al., 2016). Wang and Quake (2014) have used anti-EBV gRNAs to target the latent EBV

genome in Raji Burkitt lymphoma cells by restricting viral replication and cell proliferation. gRNAs were constructed with the purpose to target various different regions including the viral genes *EBNA1*, *EBNA3C*, and *LMP1*of the latent EBV genome. The authors reported to achieve a reduction of 65%–85% in EBV viral DNA, as well as the arrest of cell proliferation. Moreover, complete removal of the latent virus has been shown in a subset of cells that express CRISPR/Cas9 (Wang and Quake, 2014; van Diemen et al., 2016). Single gRNAs successfully resulted in up to 50% loss of EBV genomes, whereas, two gRNAs resulted in up to 95% reduction in latent EBV-positive B cells (van Diemen et al., 2016). These two studies depict that with the CRISPR/Cas9 system, the dsDNA EBV episome can be successfully targeted and removed from latently infected cells. However, *in vivo* experiments in EBV infection models, for example, humanized mice are required to analyse the therapeutic potential of this approach.

MOUSE MAMMARY TUMOUR VIRUS AND BREAST CANCER

Mouse Mammary Tumour Virus (MMTV) is a perpetuating oncogenic virus which induces a set of familiar proto-oncogenes, causing the up-regulation of the promoter region and facilitating tumorigenesis via a protein production (Schrauzer, 2006). It triggers breast cancer in mouse and transmits it either exogenously through milk, or endogenously through the germ line (Schrauzer, 2006). MMTV is also transmitted in humans from the domestic mice *Mus domesticus*, illustrating the socioeconomic impact of breast cancer in humans with high prevalence rate in poor people who live in an unhygienic environment (**Figure 1D**) (Stewart et al., 2000). MMTV sequences have been identified from the long terminal repeats (LTRs) of MMTV's genome by using PCR and NGS technologies, but with more valid outcomes from the former (**Figure 1D**) (Narty et al., 2017). By combining hybridization with DNA cloning techniques, Callahan et al. (2012) found sequences related to MMTV in human breast cancer cells. However, Szakacs and Moscinski (1991) identified the whole provirus of MMTV with a typical retroviral structure e.g. LTRs, *gags*, *pol*, *env* and RTRs in 13% of tested human breast cancers. In 2019, (Naccarato et al., 2019) identified the presence of more MMTVels in sporadic breast cancer cells than in hereditary breast cancer cells. A characteristic dUTPase in the *gag* gene has recently been discovered that is essential for viral replication and can be used for the confirmation of MMTV in human breast cancer using primer studies (Hizi and Herzig, 2015; Lehrer and Rheinstein, 2019).

MMTV has been noted to replicate efficiently in the alveolar epithelial cells of the mammary glands and an increased expression of MMTV has been observed during the lactation period due to the release of steroid hormones (Taneja et al., 2009). MMTV contains a long terminal repeat (LTR) of 1.3 kb size, which is responsible to encode a protein (super-antigens) which, in turn, activates T lymphocytes (Amarante et al., 2019). This protein triggers the replication of virus and amplification of T lymphocytes, which acts as a carrier to transfer the virus from the gut to the breast (**Figure 2C**) (Amarante et al., 2019). Moreover, the malignant transformation of human breast cancer epithelial cells is also done by the protein termed as p14 being expressed on envelope gene of MMTV (Katz et al., 2005). This p14 protein has shown its oncogenic capability when overexpressed (Feldman et al., 2012). Some other proteins like Rem, Sag, Naf or uncharacterized analogs of Tax also play a vital role in the transformation of human BC epithelial cells (Lawson et al., 2018). A virus similar to MMTV, named as the human mammary tumor virus (HMTV), which is found to be a potential cause of human breast cancer, contains DNA sequences with a 95%–99% homology with MMTV enveloped gene; it is found in samples including BC like serum, saliva, milk and others (Amarante et al., 2019). There is a consistently low level of HMTV identification in normal and benign breast cancers than human breast cancers (Lawson and Glenn, 2019). HMTV DNA sequences are linked with p53 nuclear aggregation and the presence of receptors for progesterone, which causes the cellular down-regulation, leading to breast cancer (Schrauzer, 2006).

Samples of human breast tumors have been found to include MMTV sequences from the long terminal repeat (LTR) region of the MMTV genome. These MMTV *env* gene sequences are unique to the MMTV genome and are not found in human endogenous retrovirus (HERV) sequences, which are frequently found in human genome research. In any of the MMTV-like *env* positive DNAs, no MoMt or IAP DNA sequences were found. Wnt-1 expression is much higher in MMTV-like positive breast cancer specimens than in MMTV-like negative breast cancer specimens, which is consistent with studies showing high Wnt-1 expression in MMTV positive mouse mammary tumors. PyMT, a membrane scaffold protein, activates MAPK and PI3K pathways, which are involved in cell proliferation and survival, by boosting various signaling pathways, including Shc and PI3-kinase (Cai et al., 2017; Nartey et al., 2017).

Many of the strongly enriched pathways, such as ECM-receptor interaction and focal adhesion, are essentially portion of the large "pathway in cancer" (KEGG: 05200). The PyMT oncogenesis causal route PI3K-AKT signaling is also included in the "pathway of cancer," and is connected to several of those enriched pathways. Deregulation of metabolism has long been associated with cancer, and a high-calorie diet has been shown to increase cancer risk, particularly in terms of glucose metabolism. Many metabolic related words and pathways, notably the TCA cycle pathway, were found to be overrepresented in both DEGs and WGCNA module genes (Cai et al., 2017; Nartey et al., 2017).

Although many studies have confirmed the presence of MMTV-associated breast cancer in mice, and have proposed a pathway by which MMTV can cause tumorigenesis in humans, some ambiguity is still present in this regard. Studies have reported both, the presence and absence of MMTV genome or its sequences in breast cancer samples. For example, studies of breast cancer in females only by using supplementation of Wang et al. (1995) has shown 15- fold more prevalence of MMTV-*env* sequences in human breast cancers than the control ones. Lawson and Glenn (2019) also detected 15-fold higher sequences similar to MMTV in samples of breast tumour than control samples and

reported them to be 40% more in patient samples than normal controls. Serum of women with breast cancer was also found to contain a 5-fold higher presence of MMTV antibodies than normal women serum (Lawson and Glenn, 2019). They also located the same MMTV-like sequences in lactating women and in benign breast tumour tissues 1–11 years before diagnosis (Lawson and Glenn, 2019). They reported these MMTV-like sequences to be highly similar to MMTV (Lawson and Glenn, 2017). Likewise, a study executed in Saudi Arabia also detected the presence of MMTV-*env* provirus sequences in human breast tumour samples (Al Dossary et al., 2018). However, due to heterogeneous results, it is not a definitive conclusion. There is vast difference in prevalence of MMTV- like breast cancer according to the geographical distinction. It has been observed that breast cancer prevalence is much more in western countries (30–40%) than Asian countries (10%–20%) (Stewart et al., 2000). Approximately 16% variations in Mexico's identification of MMTV breast cancers and 12% in Iraq has been observed and similar variation has been determined in United States, Italian and Australian breast cancers (Lawson et al., 2018). Furthermore, Hameed et al. (2020) declared that no association of MMTV and breast cancer was found in their study, in which they applied the Bradford Hill criteria and perused studies with both normal and benign samples along with those of breast cancer. They, however, reported that it could worsen the state of breast cancer after its tumorigenesis had already begun (Hameed et al., 2020). One more study conducted in Myanmar looked for the presence of Human Mammary Tumour Virus in breast cancer patients, which has a 90%–95% homology to MMTV and is mostly found in humans; from 58 patient samples, they found only one sample containing the HMV sequence, whose prevalence of 1.7% is obviously quite low (San et al., 2017).

Various drugs and chemicals have shown promising results in treatment of breast cancer and MMTV infection. For instance, Cathepsin D (CTSD) is a lysosomal protease marker that has shown fewer prognoses in human breast cancer by blocking tumor development in cell- independent way. Its absence delays the growth of tumor in human for 2 months. Proliferation of quiescent $CTSD^{-/-}$ tumor cells has restarted upon long term culture by reiterating oncogenic gene expression and signaling pathways (Ketterer et al., 2020). Moreover, in an ecological and correlational study, it was revealed that selenium acts as an anti-tumorigenic agent when given to MMTV positive mice as a dietary element (Schrauzer, 2006). The traces of selenium present in the samples were shown to inhibit the infection caused by MMTV in mice, whereas, chromium was found as an element antagonistic to selenium (Schrauzer, 2006). When chromium is present in +3 oxidation state, it acts as a nutritive factor, while chromium in +6 oxidation state acts as anti-selenium agent, decreasing the inhibitory effect of selenium (Schrauzer, 2006). So, chromium and selenium were showed to have interactive effects on the growth and development of mouse mammary tumor virus in MMTV infected female mice (Schrauzer, 2006). The relevance of interaction between selenium and chromium in humans is still in debate, but analysis of blood samples of workers working in a polish tannery, living in a dusty environment, and getting exposure to chromium showed decreased level of selenium (Schrauzer, 2006).

BOVINE LEUKAEMIA VIRUS AND BREAST CANCER

Bovine Leukaemia Virus (BLV) is responsible for causing bovine leucosis in cattle worldwide. It belongs to the family of Retroviridae and is closely related to human T-lymphotropic virus type-1 (HTLV-1) (Gao et al., 2020). The size of BLV ranges between 60 and 125 nm, having an enveloped capsid and a diploid positive sense RNA in its structure (Martinez Cuesta et al., 2018). In 1969, Janice Miller and co-workers first reported BLV in cattle (Buehring and Sans, 2020). It infects species close to cattle such as sheep, buffalo, and alpaca; however, the RNA genome of BLV has also been reported positive in human breast tissues (Buehring et al., 2019).

Recently a surge in female death has been observed due to breast cancer, so finding its etiological factors is necessary. In recent advanced studies, it is concluded that viruses are also a predisposing factor to cause cancer. Majorly MMTV, HPV, and EBV have been found responsible for this guilt (Lawson et al., 2018). In a few studies, BLV has also been considered a cause of breast cancer in females (Sinha, 2016). Although BLV is a zoonotic virus (found especially in cattle), some shreds of evidence show the presence of BLV in humans, too. Its presence has been detected and identified in breast cancer samples by RT-PCR, *in situ* PCR assay, ELISA, immunohistochemistry, *in situ* hybridization, and DNA sequencing (**Figure 1C**) (Khatami et al., 2020; Le et al., 2020). Most likely, the virus's presence in women's breast tissue and blood indicates its transmission from cattle to humans (Khalilan et al., 2019). Moreover, antibodies against BLV have been isolated in humans, which contribute to evidence of its transmission (Buehring et al., 2003).

The exact mechanism through which the transmission of BLV in humans takes place is unknown. However, relating some conditions can probably resolve this problem. There are several hypotheses which justify the presence of BLV in humans. According to some studies, consuming contaminated dairy products and meat of cattle positive with bovine leukemia virus could be a possible way of transmission in humans (Olaya-Galán et al., 2017). Vertical transmission has been found in cattle, which is evidence that the virus is present in their milk (Buehring et al., 2015). According to some researchers, consuming unpasteurized and undercooked meat in some areas of the world could cause transmission in humans (**Figure 1C**). Some hypothesize that before pasteurizing technique, this virus became part of the human species (Khatami et al., 2020).

Oncogene presence in a virus or activation of proto-oncogenes of cells is an essential step in converting normal cells into malignant cells. BLV neither has an oncogene, nor can it incorporate its gene into the cellular genome to activate proto-oncogene (Aida et al., 2013). Moreover, the presence of a receptor is significant for a viral infection to take place. In recent studies,

the presence of a receptor for BLV on human cells is still unknown. It indicates the resistance of humans against BLV; however, a close relation of BLV with the human T-cell leukemia virus (HTLV-1) might justify its relatedness to breast cancer (Ban et al., 1999). HTLV-1 is a human origin virus causing defects in T cells lineage. Both of these viruses have a nearly similar mode of action to cause disease.

Malignancies of the breast are not caused by directly interacting with the host genome. Instead, BLV is supposed to cause defects in the mechanisms responsible for the repair of base-pair or other kinds of mutations, leading to oxidative damage. The unrecovered mutations eventually accumulate and lead to various cancers, such as that of lungs or breast (Khatami et al., 2020).The genome of BLV is characterized by the presence of essential genes (*pro*, *pol*, *env*, *gag*), accessory genes (*G4*, *R3*) and regulatory genes (*rex* and *tax*) (Martinez Cuesta et al., 2018; Bai et al., 2020). *Tax* is also an oncogene, and is translated into proteins called Tax. This protein interferes with a number of normal functions, and renders them anomalous. The tax encoding genes are responsible for aberration in the DNA excision repair mechanism, prevention of apoptosis, and the down regulation in the function of tumour suppressors. The presence of *tax* gene, along with the *rex* gene, is marked in the Px region, which is between the envelope gene and one of the long terminal repeats (LTRs), and is a similar region present in the genome of both of these viruses (Gao et al., 2020). The presence of tax protein is also observed evidently in HTLV-1.

Tax protein can supposedly convert normal cells into malignant cells, primarily by converting lymphocytes into immortal cells. Moreover, by acting as an activating agent for cell proliferating genes, and inhibiting the repairing process of DNA damage, Tax protein can enhance uncontrolled cell division (**Figure 2D**). Studies show a new mutant form of Tax protein has been identified that is named TaxD247G. This mutant form of Tax protein can enhance genes' activation more than wild type (Aida et al., 2013). On the evidence of these functions of Tax protein, we can hypothesize that BLV can cause breast cancer in humans. Further studies are required to thoroughly understand the role of BLV in inducing breast cancer (Buehring et al., 2015).

Bovine Leukaemia Virus primarily causes infection in bovine animals or cattle. Despite this, there are studies which report the initiation of lymphosarcoma in 69 sheep ensuing contact with materials contaminated with bovine lymphosarcoma (Olsen and Baumgartener, 1976). This gives evidence that it can be responsible to commence oncogenesis in an organism other than bovine or cattle. There are also reports of it playing a causal role in tumour in other animals like rats, water buffaloes, goats, rabbits, etc. (Schwartz and Levi, 1994). Along these lines, many cases have been reported around the globe in which the presence of BLV has been confirmed in breast cancer patients. In the United States, BLV was observed in breast cancer tissues in 2015. In Brazil, using molecular and immunological techniques, BLV presence was also confirmed. A similar study conducted in Argentina indicates that BLV could also a possible cause of breast cancer. In India, a rise in milk consumption could be a likely cause of breast cancer (Gao et al., 2020). Moreover, in Australia and Colombia, BLV presence has been found (Giovanna et al., 2013; Buehring et al., 2017). In China and Japan, BLV has not been found in breast cancer samples (Zhang et al., 2016; Saito et al., 2020). The one reason which can justify the absence of virus in Chinese people is their lactose intolerance, due to which milk consumers are significantly low in China (Gao et al., 2020).

SYNERGISTIC ONCOGENIC RELATIONSHIP OF MOUSE MAMMARY TUMOUR VIRUS, HUMAN PAPILLOMA VIRUS AND EPSTEIN–BARR VIRUS IN BREAST CANCER

Many research studies show that multiple viruses co-exist in samples extracted from women afflicted with breast cancer. Glenn et al. (2012) found genome sequences of more than one virus (namely, from EBV, high-risk HPV and MMTV) co-habiting in about 72% of samples taken from women with breast cancer. Moreover, they also found that DNA extracted from 50 samples of women with invasive breast cancer contained EBV genome sequence in 68% of the samples, MMTV genome sequence in 78% of the samples, and high-risk HPV genome sequence in 50% of the samples (Glenn et al., 2012). Their results also showed that the common presence of both HPV and EBV was 38% (three- to four-fold higher) in 50 patients with breast cancer as compared to 10% of 40 normal control breast tissue samples (Glenn et al., 2012). Results of the study conducted by Naushad et al. (2017) were in concordance with the aforementioned results; PCR showed the prevalence of hish-risk HPV, EBV and MMTV genome sequences in the same samples of Pakistani women with breast cancer.

According to the studies conducted by Gupta et al. (2020) and Al Moustafa et al. (2016) co-infection of both, high-risk HPV and EBV, was found in 47% of Qatari and 32% of Syrian female breast cancer patients, respectively. Based on their own previous study conducted in 2015, Moustafa (2015) postulated that since E5 and E6/7 oncoproteins of HPV could initiate or enhance the progress of human oral carcinomas by a co-operative interaction with the LMP1 and EBNA1 oncoproteins of EBV, via the EMT event, a similar mechanism could follow the pathogenesis and metastasis of breast cancer. Another study conducted in cervical smears found that chances of HPV genome integration in host cells increased five- to seven-fold when EBV presence was found along with it, suggesting that genome instability caused by HPV was enhanced in its presence (Polz-Dacewicz et al., 2016). Moreover, it was also suggested that by producing a homolog of interleukin 10, known as the BCRF1 gene product, EBV might hinder one's immune response to cells transformed by HPV (Szostek et al., 2009). It is possible that breast cancer pathogenesis might be enhanced by any of the mechanisms suggested.

DISCUSSION

There is some evidence that Human Papilloma Virus can lead to breast cancer, but it is insubstantial. Many studies have reported both the presence and absence of HPV in breast cancer samples. For example, a study found a 26% prevalence of HPV DNA in Argentinian women with breast cancer (Suarez et al., 2013). High risk HPVs, namely HPV 16, 18 and 31, were also detected in 16 of 72 Egyptian females afflicted with breast cancer (El-Sheikh et al., 2021). However, a study conducted in Indian women with breast cancer found no traces of either HPV DNA or E6 and E7 proteins, both, by conventional and real time PCR (Hedau et al., 2011). Similarly, another study done on patients with breast cancer in Iran found DNA of HPV in only eight out of the 98 patient samples, which is an extremely low prevalence; among HPV positive samples, 62.5% of DNA was of HPV 16 and 18 (Malekpour Afshar et al., 2018). In addition, Kwong et al. (2013) found no traces of HPV by PCR in their study of 102 breast cancer patient samples in Hong Kong.

Most of the accounts aforementioned reporting the presence of HPV genome in breast tumour samples is in Western females. There is also a higher viral load in patients of breast cancer as compared to controls. On the contrary, absence of HPV DNA or extremely low prevalence is mostly seen in Eastern particularly, Asian, females. Therefore, to get a better picture of the causal link between HPV and breast cancer, more studies should be conducted on Eastern women with breast cancer, especially those who were previously affected by HPV infections in their life.

Similar results have been reported in regard to EBV. A study conducted in France discovered that 51% of tumour samples contained the EBV genome, while 90% of healthy tissue samples taken from near the tumour site showed its absence (Bonnet et al., 1999). This indicates that EBV genome is significant in these oncogenic epithelial cells, as neighbouring normal cells showed no trace of it. Moreover, Zekri et al. (2012) detected the presence of EBV in 45% of Egyptian women with breast cancer and 28% of Iraqi breast cancer patients and also reported a more plausible relationship of EBV with increased aggressiveness of the cancer. Meanwhile, Dowran et al. (2019) found no traces of EBV in Iranian breast cancer patients. Likewise, Kadivar et al. (2011) found an absence of EBV in 100 breast cancer patient samples.

Most of the studies which support the hypothesis that breast can also be EBV associated are conducted in the West. On the other hand, reports which deny its association with breast cancer and present mostly in Asia. However, no conclusive statement can be given in this regard, as any EBV-associated breast cancer samples were not found in America and New Zealand, too. It is possible, though, that breast cancer can be worsened or become aggressive by the infection of EBV after tumorigenesis. In any case, more studies need to be executed to eliminate this conflicting result.

In concordance with HPV and EBV, there is substantial evidence that MMTV can cause cancer in human breast tissues, but the underlying mechanism which execute this are not yet clear and defined. The evidence reports includes a study carried out in Egypt aimed to detect the presence of *env* sequences in MMTV-like DNA in samples of familial and non-familial breast cancer; the prevalence they found was 70% in familial and 76% in non-familial women suffering from breast cancer (Loutfy et al., 2021). Furthermore, one more study reported 65.72% prevalence of MMTV sequences on 105 breast cancer patients and also suggested that be used as a biomarker for cancer invasion (Khalid et al., 2021). However, a study reported a contrasting result; they detected the presence of MMTV in human breast cancer samples, but due to inconsistency and a later confirmation of it being murine DNA, they concluded that no association was present between MMTV and human breast cancer (Perzova et al., 2017). Likewise, another study which took place in Iran reported that they found no traces of two sequences of MMTV-like DNA by nested PCR in any of their 300 breast cancer patient samples (Motamedifar et al., 2012).

As compared to HPV and EBV, more studies reported the presence of MMTV or MMTV-like sequences in breast cancer in Western countries as compared to Eastern countries. Moreover, since some studies enumerate that an increased frequency of MMTV-like sequences were found in patient samples as compared to control, suggests that it may enhance tumour induction. However, this gap in these findings certainly needs to be abridged to get a picture with more clarity. A concrete statement cannot be given with such contrasting studies also existing, and being continuously reported, around the world. Therefore, it is more prudent to report that MMTV or MMTV-like sequences may play causal role for breast cancer, but evidence regarding this is still inconclusive.

BLV-associated infection and cancer is usually found in bovine animals and cattle, but varying results regarding it are also seen in human breast cancer samples. For instance, a case control study carried out in Texas, America, reported a significant presence of BLV genome in tissues of women with breast cancer as compared to those women with benign tumour or were normal (Baltzell et al., 2018). To know if there was any associative relationship of BLV and HPV in causing malignant breast cancer, they also executed PCR and DNA hybridization tests; however, they found no traces of HPV in any of the samples (Baltzell et al., 2018). Similarly, Khatami et al. (2020) reported by their meta-analysis study that nine case control studies centring on the causal role of BLV for breast cancer confirm the notion. As aforementioned, some reports also propose contrasting results. This could be due to low milk consumption in the Chinese and Japanese population, as compared to other countries where there was a prominent association between BLV and breast cancer, as already mentioned above.

Collective survey of literature does suggest that an association between BLV and breast cancer is highly likely, but the evidence is still insufficient. Efforts to eradicate bovines, cattle and other animals from BLV might be one way to control its supposed effect on breast cancer.

CONCLUSION

In our opinion, even though there is evidence that Human Papilloma Virus can cause cervical and oral cancers and is considered as a high risk factor for breast cancer, it is not

sufficient to conclude that it alone is a causal factor for breast cancer. Moreover, evidence that Mouse Mammary Tumour Virus can lead to breast cancer is suggestive and comprehensive, but is not conclusive because its prevalence is higher in Western females as compared to women residing in the East. In addition, although a pathway leading to the pathogenesis of breast has been proposed for Epstein – Barr Virus, it is inadequate to conclude that it is one of the root factors for breast cancer. Similarly, even though there are suggested pathways by which Bovine Leukemia Virus can confer breast cancer and is suggested to be highly likely, it is insufficient to report it as conclusive, due to different reports in varying geographical distributions. However, evidence that presence of multiple viruses in specimens of breast cancer, is more detailed and suggestive. More studies are required to conclude that these viruses definitely lead to the malignancy that is breast cancer. Moreover, even if there is a definite causal relationship between viruses and breast cancer, control of these viral infections in the first place might curb reported cases.

AUTHOR CONTRIBUTIONS

KF: Writing- Draft preparation, reviewing and editing. AN: Writing- Draft preparation. AS: Writing- Draft preparation. AH: Writing- Draft preparation. AB: Writing- Draft preparation. MA: Writing- Draft preparation and graphics designing. SN: Writing- Draft preparation. SZ: Writing- Draft preparation. SA: Writing- Draft preparation. SH: Writing- Draft preparation. MS: Reviewing. SA: Reviewing and supervision. All authors listed have made a substantial and intellectual contribution to get this article approved for publication.

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frontiers | Frontiers in Molecular Biosciences

REVIEW
published: 14 April 2022
doi: 10.3389/fmolb.2022.783494

'Breast Cancer Resistance Likelihood and Personalized Treatment Through Integrated Multiomics'

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In recent times, enormous progress has been made in improving the diagnosis and therapeutic strategies for breast carcinoma, yet it remains the most prevalent cancer and second highest contributor to cancer-related deaths in women. Breast cancer (BC) affects one in eight females globally. In 2018 alone, 1.4 million cases were identified worldwide in postmenopausal women and 645,000 cases in premenopausal females, and this burden is constantly increasing. This shows that still a lot of efforts are required to discover therapeutic remedies for this disease. One of the major clinical complications associated with the treatment of breast carcinoma is the development of therapeutic resistance. Multidrug resistance (MDR) and consequent relapse on therapy are prevalent issues related to breast carcinoma; it is due to our incomplete understanding of the molecular mechanisms of breast carcinoma disease. Therefore, elucidating the molecular mechanisms involved in drug resistance is critical. For management of breast carcinoma, the treatment decision not only depends on the assessment of prognosis factors but also on the evaluation of pathological and clinical factors. Integrated data assessments of these multiple factors of breast carcinoma through multiomics can provide significant insight and hope for making therapeutic decisions. This omics approach is particularly helpful since it identifies the biomarkers of disease progression and treatment progress by collective characterization and quantification of pools of biological molecules within and among the cancerous cells. The scrupulous understanding of cancer and its treatment at the molecular level led to the concept of a personalized approach, which is one of the most significant advancements in modern oncology. Likewise, there are certain genetic and non-genetic tests available for BC which can help in personalized therapy. Genetically inherited risks can be screened for personal predisposition to BC, and genetic changes or variations (mutations) can also be identified to decide on the best treatment. Ultimately, further understanding of BC at the molecular level (multiomics) will define more precise choices in personalized medicine. In this review, we have summarized therapeutic resistance associated with BC and the techniques used for its management.

Keywords: breast cancer, drug resistance, genomics, transcriptomics, proteomics, metabolomics, radiomics

OPEN ACCESS

Edited by:
Abbas Khan,
Shanghai Jiao Tong University, China

Reviewed by:
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Specialty section:
This article was submitted to Molecular Diagnostics and Therapeutics, a section of the journal Frontiers in Molecular Biosciences

Received: *26 September 2021*
Accepted: *14 March 2022*
Published: *14 April 2022*

Citation:
Mehmood S, Faheem M, Ismail H, Farhat SM, Ali M, Younis S and Asghar MN (2022) 'Breast Cancer Resistance Likelihood and Personalized Treatment Through Integrated Multiomics'. Front. Mol. Biosci. 9:783494. doi: 10.3389/fmolb.2022.783494

INTRODUCTION

Cancer is a common disease and represents one of the biggest health problems in the world and a significant global concern. The incidence and mortality rates of breast cancer (BC) have increased in recent years, and BC is currently the leading cause of cancer deaths in women worldwide.

According to the global cancer statistics in 2020, breast cancer (BC) in women was reported as the primary leading cause of deaths (Bray et al., 2018; WHO 2021). It occurs in every country of the world and in women of every age, although later years of life are an increased risk factor. According to the WHO fact sheet on breast cancer, in 2020, 2.3 million women were diagnosed with breast cancer, and 685,000 died from this cancer. As estimated at the end of 2020, almost 7.8 million women have been diagnosed with breast cancer in last 5 years (WHO 2021). This has made breast cancer the most prevalent cancer globally, and its prevalence has even surpassed lung cancer, which was previously the highest diagnosed cancer (Sung et al., 2021).

Breast cancer (BC) mainly has four molecular subtypes which have been defined in the large part of the hormone receptor or other forms of protein involved or not involved in each type of cancer: 1) luminal A or HR+/HER2– (HR-positive/HER2-negative) 2) luminal B or HR+/HER2+ (HR-positive/HER2-positive) 3) HER2-positive 4) triple-negative or HR–/HER2– (HR/HER2-negative) (Eliyatkın et al., 2015). This classification is mainly based on the type, behavior, and pattern of the cancer cells. A comprehensive understanding of all these types enabled the researchers and scientists to develop the targeted treatments and also the understanding that which type of treatment is best suited for which type of cancer cells (Sharma et al., 2010). Among all of the aforementioned types, the triple-negative subtype is the most prevalent and most aggressive as its response to chemotherapy is quite higher than that of the other types. Moreover, despite adjuvant chemotherapy, the survival rate of the patients with the triple-negative type is very poor (Anders and Carey 2009).

Chemoresistance is the major problem in the treatment and management of BC, when there is a relapse in the early-responsive tumors and development of resistance toward the multiple anticancer agents having various mechanisms and structures (Perez, 2009). Chemoresistance of tumors can be associated with multiple factors or mechanisms, which include its microenvironment, interaction with other cancer cells, modulation of immune cells and macrophages associated with cancer cells, cancer stem cells, and heterogeneity of cancer cells, that can modify the microenvironment of the cancer cells or tumors during chemotherapy which can lead to the development of resistance in them. There are several intrinsic factors contributing toward resistance development including the pH of cells, paracrine signaling among cells, and the hypoxia environment (Mansoori et al., 2017; Nikolaou et al., 2018). Another type of resistance toward multiple anticancer agents is known as multidrug resistance (MDR). However, the potential role of the drug-resistant genes that are involved in the transportation of anticancer agents is still unclear. Therefore, a clear understanding of the underlying mechanism of chemotherapy resistance and available treatments is required to develop successful strategies to overcome multiple drug resistance and other chemotherapy-associated resistances (Wind and Holen 2011).

There are many types of treatment therapies (surgery, chemotherapy, hormonal therapy, biological therapy, and radiation therapy) available depending upon the type of the cancer cell (Division of Cancer Prevention and Control, 2020). To choose the treatment for BC, there are certain modalities that need to be considered such as the location and size of the tumor, histopathology, lymph node commitment, presence or absence of metastases, and the molecular subtype of the cancer cells. Moreover, patient age, health, and hormonal status should be taken into consideration (NCI, 2022), Cardoso et al., 2019). Although chemotherapy has been used for the treatment of inflammatory and advanced-stage BC, there is a need to develop new strategies and predictive molecular markers to increase the prognosis of the patients (Cleator et al., 2007). The unpleasant side effects of the available breast cancer treatment methods motivate researchers to find some alternative options (Akram et al., 2017). The development of precision medicine is a great hope toward better breast cancer management. The precision medicine refers to the consideration of individual variations, environment, genes, and lifestyle for disease prevention and treatment (Collins and Varmus 2015). The recent advancement in the omics technology has allowed a more precise approach toward breast cancer treatment (Naito and Urasaki 2018). Moreover, the novel prognostic and predictive markers will be helpful in determining the patient that could benefit from the chemotherapy. In addition, different strategies can be defined to increase the targeted drug delivery response toward tumor cells which includes nanoparticles as well. These small nanostructures can be effective carriers not only in chemotherapy but also to overcome drug resistance as well (Lainetti et al., 2020).

BREAST CANCER RESISTANCE LIKELIHOOD

Breast cancer is a very complex and heterogeneous disorder with unique molecular and morphological features relative to a disease which involves only a single gene or protein in a simple signaling pathway contributing toward the progression of disease in an independent and autonomous manner (Organization 2019). Various studies had represented BC heterogeneity through the differential response of the same type of BC patients to treatment and risk of developing side effects. One of the major clinical complications in the treatment of breast carcinoma patients is the development of therapeutic resistance (Luque-Bolivar et al., 2020). Recently drug resistance in BC treatment is not properly addressed, rather to focus on molecular pathways deeply; an alternative strategy of using a different drug is commonly applied. In order to reduce the adverse effects of BC treatment including drug resistance, a profound understanding of the molecular mechanism of the disease and the response to the drug is needed. Multidrug resistance (MDR)

TABLE 1 | Overview of drug resistance to various BC subtypes and alternative approaches to overcome resistance.

BC subtype	Treatment options (drugs)	Drug resistance	Resistance treatment options	References
ER+	A selective ER modulator, tamoxifen (TAM)	1. Mutations in estrogen receptor 1 (*ESR1*) gene and polymorphisms in cytochrome P450 family 2 subfamily D member 6 (*CYP2D6*) cause disruptions in TAM metabolism	Selective ER downregulator (fulvestrant, FUL) treatment is applied which has relatively low toxicity than TAM	(Kang et al., 2005
		2. Alterations in translation signals due to aberrant activation of cyclic adenosine monophosphate/protein kinase A (cAMP/PKA), mitogen-activated protein kinase (MAPK/ERK), and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathways		Li et al., 1997
		3. Mutations in the tumor suppressor protein, phosphatase, and tensin homolog (PTEN) may lead to activation of the PI3K/AKT pathway which causes TAM resistance		Group 2011
				Zakharchenko et al., 2011
				Razavi et al. (2018)
ER+/PR+/HER2-	selective ER downregulators (fulvestrant, FUL)	Mutations in the *PIK3CA* gene	Combination with Piqray (alpelisib) (FUL + Piqray)	(Thorpe et al., 2015
				Administration (2019)
All clinical stages of BC	Aromatase inhibitors (AIs) (anastrozole, exemestane, and letrozole)	Relapse after initial treatment with a non-steroidal AI (anastrozole or letrozole)	Treatment with exemestane alone or in combination with an mTOR inhibitor such as everolimus	(Carlini et al., 2007
				Chin et al., 2007
				Geisler et al., 2008
				Bahrami et al. (2020)
ER+/HER2-	CDK4/6 inhibitors (palbociclib, ribociclib, and abemaciclib)	Uncomplicated and manageable hematological mainly neutropenia and non-hematological toxicities with dose interruption or reduction	Combination of CDK4/6 inhibitors with FUL and AI's	(Asghar et al., 2015
				Turner et al., 2018
				Killock 2019
				Rossi et al. (2019)
ER+	*PI3K* inhibitors	Higher toxicity from the FUL + pictilisib combination treatment	Overcomes resistance to hormone therapy by controlling the AKT/mTOR signaling pathway by using everolimus (Afinitor) with the CDK4/6 inhibitor	(Hurvitz and Peddi 2013
	Pictilisib and buparlisib			Krop et al., 2016
				Dhakal, Antony Thomas et al. (2020)
HER2+	Humanized monoclonal antibodies (mAbs) trastuzumab, pertuzumab, and 19H6-Hu	Truncated form of HER2 (P95HER2) through proteolytic detachment created clinical resistance to trastuzumab	1.Pertuzumab is a second-generation recombinant humanized monoclonal antibody that binds to the extracellular dimerization domain II of HER2	(Warmerdam et al., 1991
		PIK3CA mutations	2. A new anti-HER2 antibody (19H6-Hu), which enhances the antitumor efficacy of trastuzumab and pertuzumab with a distinct mechanism of action	Christianson et al., 1998
		FCGR	3. PI3K/AKT/mTOR inhibitors along with trastuzumab or trastuzumab and paclitaxel are efficient and more safe	Quandt et al., 2011
		IIa polymorphisms		Administration 2019
				Zhang et al. (2020)
TNBC	Chemotherapy by alkylating agents, antimetabolites, anti-tumor antibiotics, topoisomerase inhibitors, TKIs, and mitotic inhibitors.	Alterations in the epigenetic mechanism	Epigenetic therapies, such as hydralazine and valproic	(Verweij et al., 1994
		Enzyme system that deactivates anticancer drugs	Neoadjuvant chemotherapy and taxanes along with anthracyclines	Miklavčič et al., 2014
		Tumor microenvironment, upregulation of *TWIST1* by NF-κB contributes to the chemoresistance	Immunotherapy	Loibl and Furlanetto 2015
			ECT	Jazieh et al. (2020)

and consequent relapse on therapy are prevalent issues related to breast carcinoma as our understanding is incomplete related to the molecular mechanism of breast carcinoma disease (Waks and Winer, 2019a). Therefore, elucidating the molecular mechanisms involved in drug resistance is critical. For the management of breast cancers, the treatment decision not only depends on the

assessment of prognosis factors but also on the evaluation of pathological and clinical factors. Integrated data assessments of these multiple factors of breast carcinoma through multiomics can provide significant insight and hope for making therapeutic decisions (Parsons and Francavilla 2020). Major BC treatment strategies rely on the tumor subtype, immunohistochemical evaluation of prognostic elements, and seek new genetic markers to improve the diagnostic strategies and to enhance treatment outcomes with minimal side effects.

CONVENTIONAL BREAST CANCER TREATMENT RESISTANCE

Endocrine therapy is included in one of the key conventional BC treatments along with chemotherapy and targeted therapy. For instance, it is used for treating tumors with positive hormone receptors (ER and PR) (luminal A and luminal B); however, chemotherapy is also required for some patients. Monoclonal antibody treatment is applied for HER2+ tumors (luminal B and HER2+). For positive hormone receptors, RNAi-mediated silencing and endocrine therapy are helpful (Tai et al., 2010; Harbeck et al., 2019; Waks and Winer, 2019b). The triple-negative breast cancer (TNBC) is only treated with chemotherapy. Various molecular players are being explored to study the BC cell resistance development to conventional therapies. Both intrinsic and extrinsic factors contribute toward creating resistance by BC cells, including its microenvironment, interaction with other cancer cells, modulation of immune cells and macrophages associated with cancer cells, cancer stem cells, and heterogeneity of cancer cells, that can modify the microenvironment of the cancer cells or tumors during chemotherapy which can lead to the development of resistance in them. There are several intrinsic factors contributing toward resistance development including the pH of cells, paracrine signaling among cells, and hypoxia environment. Another type of resistance toward multiple anticancer agents is known as multidrug resistance (MDR). A detailed overview of drug resistance to various BC subtypes and alternative approaches to overcome resistance is represented in **Table 1**. Here, out of many conventional treatment options, endocrine therapy is taken as a standard therapy for the treatment of ER+ BC, which includes the use of selective ER modulators, such as tamoxifen (TAM) (Group 2005; Cardoso et al., 2019) selective ER downregulators (fulvestrant, FUL), and aromatase inhibitors (AIs).

OVERCOMING RESISTANCE THROUGH EMERGING TECHNOLOGIES

In recent years, advances have been made in BC treatment options including immunotherapy, cyclin-dependent kinase 4/6 (CDK4/6) inhibitors, tyrosine kinase inhibitors (TKIs), clustered regularly interspaced short palindromic repeats (CRISPR), microRNAs (miRNAs), use of already available drugs for different diseases for treating BC (drug repurposing), nanotechnology-based treatments, and electrochemotherapy (ECT). Additional benefits have been added to the conventional treatment options by introducing new strategies in terms of decreasing the side effects and overcoming resistance. Multiomics is the most recent emerging technology for treating BC through personalized decisions and treatment options. It usually generates a vast amount of data on different kinds including genomics, transcriptomics, proteomics, metabolomics, and radiomics. One of the biggest challenges is to integrate in data to obtain biologically meaningful insight (Wang et al., 2014; Nik-Zainal et al., 2016). For this purpose, researchers need robust and sophisticated computational systems to integrate and analyze the data in a standardized manner (Chen et al., 2017). This can be achieved by making improvements in technology for better results in less sample processing and measurement time.

The BC treatment decision not only depends on the assessment of prognosis factors but also on the evaluation of pathological and clinical factors. Integrated data assessments of these multiple factors of breast carcinoma through multiomics can provide significant insights and hope for making therapeutic decisions. The implementation of omics approaches including genomics, transcriptomics, proteomics, metabolomics, and radiomics in clinical practice will assist the analysis of global level patient's changes which improves diagnosis and therapeutic choice on the basis of few markers (Scherf et al., 2000; Staunton et al., 2001; Bild et al., 2006). Early tumor detection will be facilitated by identification of omics technology-guided biomarkers, ultimately leading to early treatment and management of disease as marking the novel molecular targets confined to specific BC subtypes will decrease the reliance on non-targeted therapies, thus improving the quality of life for breast cancer patients.

MULTIOMICS APPROACHES AND BREAST CANCER MANAGEMENT

Multiomics also described as panomics and/or integrative omics is an analytical approach that combines data from multiple 'omics' approaches including genomics, transcriptomics, proteomics, metabolomics, epigenomics, metagenomics ,and metatranscriptomics to answer the complex biological questions. This omics approach is particularly very helpful in identifying biomarkers of health, disease, and treatment progress by collective characterization and quantification of pools of biological molecules within and among the cells. A range of omics software and databases are available for this analysis. Omics techniques produce a large amount of the data which is then processed. Advanced technologies have allowed 'omics' data analysis in a combined, interconnected, and holistic format to solve the complex biological problems which could not have been found with experimental work in the laboratory **Figure 1**. Systems biology is an approach in biomedical research to understand the larger picture be it at the level of the organism, tissue, or cell by putting its pieces together. It is in stark contrast to decades of reductionist biology, which involves taking the pieces apart.

GENOMICS AND BREAST CANCER MANAGEMENT

NGS has allowed rapid DNA sequencing covering the whole genome. This approach helped in redefining the breast cancer subtypes and identification of mutations and SNPs as biomarkers for BC management (Parsons and Francavilla 2020). Additionally, single-cell investigation allowed the study of BC stem cells as a novel therapeutic approach (Lawson et al., 2015). Genomics has started to change the trend of BC treatment. Genomics with molecular signatures deescalated chemotherapy and personalized treatments of BC. Molecular signatures play vital roles in the prediction of therapeutic targets. In BC, key signatures are the PR (progesterone receptor), ER (estrogen receptor), and HER2 (human epidermal growth factor receptor 2) (Rakha et al., 2010). For management, if a patient is PR+ or ER+ will probably receive endocrine treatment, while HER2 patients will likely receive trastuzumab. Triple-negative breast cancer (TNBC) covers all types of tumors which are PR-, ER-, and HER2-negative. TNBC is a more aggressive tumor and is associated with a poorer outcome to chemotherapy. However, there is still no targeted therapy for TNBC (Foulkes et al., 2010).

In the context of hereditary predisposition, the United States National Comprehensive Cancer Network proposed 19 genes as clinical screening tests for BC, while the Genetics and Cancer Group proposed 13 genes for prevention and screening measures (Hamdan et al., 2019). Genome sequencing enables the discrimination of genetic modifications on the basis of *TP53*, *PIK3CA*, and *GATA3* genes, and results suggested that these genes are modified in more than 10% of BC patients. On the other hand, NGS revealed that BC generally carries mutations in the *TP53*, *BRCA1*, and *RB1* genes (Koboldt et al., 2012). It is estimated that *BRCA1* mutations chances are in 10% of patients. However, in young females, TNBC chances are 20% (Peto et al., 1999). *BRCA1* mutations do not account for all inherited BC cases associating the existence of other genes (Ellsworth et al., 2010). *BRCA1* and *BRCA2* identification opened the paths for screening tests to identify different mutation points for hereditary BC. For early age diagnosis, BC screening is now recommended for females with a family history of cancer (Nelson et al., 2005). Currently, BRACAnalysis® is the sole sequencing provider for the detection of mutations in *BRCA1* and *BRCA2* (Ellsworth et al., 2010).

A meta-analysis of genomic studies recognized 84 loci, probably associated with the risk of BC including lymphocyte-specific protein (LSP1), fibroblast growth factor receptor-2 (FGFR2), mitogen-activated kinase-1 (MAP3K1), and trinucleotide repeat containing 9 (TNRC9/LOC643714) (Ellsworth et al., 2010; Michailidou et al., 2015). Along with it, several low penetrance variants were also identified without any validation. One such variant is the FGFR2 oncogene whose protein is being highly expressed in 5% of BC patients. This refers to SNP which affects the target binding site of FGFR2 and activates the additional downstream pathway (Moffa and Ethier 2007). Similarly, another SNP, in the 8q24 region, regulates the C-MYC oncogene (Ahmadiyeh et al., 2010).

Ki67 is another proliferative biomarker that is currently being used to predict the growth rate of tumor (Lal et al., 2017). The combination of these four signatures (ER, Ki67, PR, and HER2) is referred to as a protein-based 'signature'. On the basis of this panel, different algorithms have been developed for the prediction of the BC recurrence risk. Several models have been validated to enhance the BC management with a combination of pathological, clinical, and biosignature data. Numerous tools have been designed (e.g., Predict, Online, Adjuvant!, and the Nottingham Prognostic Index) to help clinicians with patients' treatment decision about adjuvant therapy or surgery. These tools incorporate various pathological and clinical variables together with the tumor expression of these molecular signatures (ER, Ki67, PR, and HER2) to predict survival with or without adjuvant therapy (Bartlett et al., 2016). In spite of recent achievement in identifying genetic biomarkers with additional low-risk alleles and low frequency, highly-incident variants (Bodmer and Bonilla 2008) and environmental interactions with genes must be evaluated, and methods must be established to assess mechanisms by which DNA variants in intronic or intergenic regions contribute to BC (Ellsworth et al., 2010).

TRANSCRIPTOMICS AND BREAST CANCER MANAGEMENT

The study of the complete set of RNA molecules that are produced by the genome under specific conditions in specific cell/tissue using modern techniques, e.g., microarrays and RNA-Seq, fall under the umbrella of transcriptomics. Transcriptomics has been widely used to investigate biomarkers for BC's risk assessment, subtype identification, disease progression, survival, and invasion that could be subsequently utilized to assess treatment success and clinical trials (Transcriptome 1–4). In the breast cancer treatment, biomarkers are crucial as prognostic or predictive properties. Based on the values of these biomarkers, BC treatment which could be hormonal therapy, chemotherapy, and molecular targeted therapy is planned. Transcriptomics has assisted a lot in the discovery of the BC's biomarkers. In the subsequent section, we have discussed a few of the biomarkers that have been discovered and are used for BC's management.

Transcriptome-wide association studies (TWAS) combine the data from whole genome sequencing and microarray or RNA-Seq to get insights into the BC's management. Mancuso et al. identified 1,196 genes that were associated with 30 complex biological pathways in BC using the TWAS approach (Mancuso et al., 2017). At present, three TWAS studies have been reported by different groups. Gao et al. reported TP53INP2 (tumor protein p53-inducible nuclear protein 2) to be efficiently linked with ER-negative BC in all three studied populations, i.e., African, European, and Asian ancestry populations (Gao et al., 2017). Similarly, Hoffmann et al. identified significant links between the BC risk and the expression of RCCD1 (RCC1 domain containing 1) and DHODH (dihydroorotate dehydrogenase) in the breast tissue, along with association with ANKLE1 (Ankyrin Repeat and LEM Domain Containing 1) in trans-ethnic meta-analyses of U4C, and UK Biobank data were elucidated (Hoffman et al., 2017). Wu et al. identified 48

genes from which 14 were novel using the data acquired for the Genotype-Tissue Expression Project. The effect of these genes on cell proliferation and colony-forming efficiency was elucidated to provide insights into the BC biology (Wu et al., 2018). Another group identified 26 new target genes for breast cancer including 17 genes for estrogen receptor (ER)-negative BC using expression quantitative trait loci (eQTL). Furthermore, seven regions with variants linked with BC risk and four regions for ER-negative BC risk were also identified *via* gene-based test of linkage that considers eQTL from multiple tissues. However, the function of most of these genes was not known (Ferreira et al., 2019). These studies have reported 59 genes whose predicted expression levels are associated with a high risk of BC. Additional five genes are associated with the ER-disease risk. Of these 64 genes, 30 are at loci that were not previously identified by breast cancer GWAS.

Feng and co-workers identified two genes, *HIST2H2BA* and *STXBP4*, which were precisely associated with ER+ but not with ER- BC through meta-analysis using publicly available data for whole transcriptome and genome sequencing from the GTEx database. Furthermore, 26 old and four novel biomarkers were also identified that were associated with BC's risk (Feng et al., 2019).

Currently, six tests including the Breast Cancer Index, EndoPredict, MammaPrint, Oncotype DX, Prosigna, and genomic grade index have been designed on the basis of the transcriptomic signatures for early diagnosis of the BC. The breast cancer index is designed on 60 ER+ tumor samples from patients previously treated with tamoxifen. It measures the ratio of *HOXB13* and *IL17BR* genes together with expression of the genomic grade index genes including *BUB1B*, *NEK2*, *CENPA*, *RRM2*, and *RACGAP1*. This test is used to determine the prognosis of the women with estrogen receptor-positive and lymph node-negative disease (Ma et al., 2008; Jerevall et al., 2011). The EndoPredict is designed on 964 ER+ tumor samples from patients with LN ± disease treated with tamoxifen. This test includes the expression of eight tumor-associated genes including *BIRC5*, *UBE2C*, *RBBP8*, *AZGP1*, *IL6ST*, *MGP*, *DHCR7*, and *STC2* and three control genes *OAZ1*, *CALM2*, and *RPL37A*. This test is used in determining the prognosis of women with estrogen receptor-positive and lymph node ± disease (Filipits et al., 2011).

MammaPrint is a 70-gene test which uses microarray technology for quantitative expression of the genes belonging to the following processes: cell-cycle dysregulation (15 genes), angiogenesis (12 genes), proliferation and oncogenic transformation (11 genes), invasion and metastasis (8 genes), growth factor signal transduction (6 genes), resistance to apoptosis (2 genes), and miscellaneous/unknown function (16 genes). This test has been designed on 78 ER ± tumor samples with a diameter. This test determines the prognosis of women with ER ± and LN− disease of stages 1 or 2. This assay was approved in 2007 by the FDA to predict the risk level of a patient for developing metastasis. (Verweij et al., 1994; Van't Veer et al., 2002). Oncotype DX has been evaluated on 447 ER ± tumor samples from patients with LN ± disease registered in three distinct clinical trials, including from the tamoxifen only the arm of NSABP B-20. This test measures genes for proliferation (5), invasion (2), estrogen (4), HER2 (2), GSTM1, BAG1, CD68, and also five genes for reference. It is used to predict 10-year recurrence risk in patients with ER+ and LN− disease (Paik et al., 2004). The Prosigna test is designed on 189 ER ± tumor samples from patients with LN ± disease and 29 nonmalignant breast tissue biopsy samples. This test measures the expression level of 50 genes along with five reference genes to classify BC into one of four intrinsic subtypes. Clinically, it has been utilized to also determine the prognosis of postmenopausal women with ER+ and LN ± disease of stages 1 or 2 (Parker et al., 2009; Nielsen et al., 2010) Although ample work has been done on the discovery of the biomarkers for BC's diagnosis, progression, and treatment end point, further investigations are required to identify the biomarkers for diverse forms of the breast cancer.

PROTEOMICS AND BREAST CANCER MANAGEMENT

Proteomics is the fine study of complete set of proteins present in any tissue, cell, or organism. Breast cancer (BC) proteomics research is based on validating and discovering protein predictive biomarkers diagnostic purposes. Recently, a study of four groups reported the survival patterns of BC functional proteins (Korkola and Gray 2010) which revealed about 10 different protein biomarkers that might differentiate BC subgroups biologically and clinically more accurately as compared to prognostic markers. Umar et al. (2005) identified nine tryptic peptides being differentially expressed by stromal and tumor analysis using laser capture microdissection. Afterward, Sanders et al. (2008) reported the reduced expression level of S100-A8 and ubiquitin in BC tissue than that in normal tissue.

Mass spectrometry analysis of the BC proteome revealed that protein-specific patterns are responsible for early diagnosis. 14-16 MS analysis also identified different peptide biomarkers including fragments of C3, C3adesArg, factor XIIIa, ITIH4, FPA, apoA-IV, fibrinogen, bradykinin, and transthyretin. These biosignatures can be used as a landscape for the early diagnosis of BC. Palacios et al. (2008) reported 37 protein biomarkers using proteomics classification. Among these, BRCA2-mediated cancers are found to be associated with the D1 and D3 cyclins along with CDK4. In another study, using protein markers and signaling pathways, five subtypes of ER-positive BC have been reported consisting of normal, basal, overexpressed HER-2, luminal A, and luminal B (Reis-Filho and Tutt 2008; Qin and Ling 2012; Zeidan et al., 2015). Collectively, 97 BC biosignatures have been reported so far from pathological and proteomics studies including ER, p53, CK8/18, Ki-67, PR, cyclin D1, HER-2, CK5/6, cyclin E, BCL2, cyclin E, and E-cadherin (Bhargava et al., 2008; Qin and Ling 2012; Zeidan et al., 2015). In another proteomic study, scientists have reported the role of retinoic acid receptor alpha as a potential biosignature in 28 ER-positive patients. Brozkova et al. identified the proteomic role of HSP27 and ANXV as biomarkers in BC 21, 22. He et al. (He et al., 2013) by using MS and ELISA reported that serum CD14 could be an active biomarker for the prediction of BC.

Kabbage *et al.* reported the overexpression of the Hsp27 and Hsp5 in BC tissues which are known as α-B-crystallin. Moyano *et al* (Moyano et al., 2006) reported that α-B-crystallin can solely be responsible for cancer transformation because it can induce the expression of EGF and anchorage-independent growth. α-B-crystallin has the ability to enhance cell invasion and migration along with the activation of the MAPK/ERK pathway. These reports suggest the oncoprotein nature of α-B-crystallin. Li et al. (2005) investigated three protein signatures including one reducing protein (4.3 kDa) and two increasing proteins (8.1 and 8.9 kDa) for BC. Studies revealed that structurally these proteins consist of the ITIH4 chain, C3adesArgΔ8 peptide, and C3adesArg (Belluco et al., 2007).

Hudelist et al. (2006) performed MALDI-TOF and 2-DE comparative analysis of LCM from normal and tumor tissues of five BC patients. In normal tissues, proteins with high MW and low isoelectric points were expressed in the extracellular matrix, while in LCM tissues, proteins with intermediate MW and high isoelectric points were overexpressed. Collectively, 32 proteins were expressed differentially and identified as tumor-suppressor genes, cytokines, signal-transducers, structural proteins, and cell-cycle regulators. Some proteins suggest their active role in tumor suppression as they are subregulated during cancer invasion including Maspin, DCC, and DSG3. On the other hand, CATH, HER-3, and HSP-27 are overexpressed during cancer invasion. Some overexpressed proteins such as CGG3 have a significant role in malignant transformation in BC also termed as ALADIN (Fink-Retter et al., 2009).

Pietrowska et al. (2010) reported proteome analysis in frozen LCM of breast tumor using MALDI MS. He compared protein expression in ER-negative and ER-positive tumors along with invasive carcinoma in mammary epithelium. Biosignatures were identified using appropriate statistical models and classifiers were validated in blinded tests. They used LC-MS/MS for identification and IHC for the confirmation of m/z features of the classifiers. A group of scientists compared the level of ubiquitin and calgranulin-A in 167 normal tissues with 122 tumor tissues, and it was found that ubiquitin expression was decreased while the expression of calgranulin-A was enhanced in tumor tissues. This study led to the identification of three biosignatures for BC. Schulz et al. (2009) reported the proteomic expression of TNBC compared with Her-2 positive tumors using MALDI-TOF/MS and 2D-DIGE. Through this technique, vimetin, L-plastin, glycolytic enzymes, fironectin, cytokeratins, annexin-1, annexin-2, and peroxiredoxin proteins were identified and validated by IHC and Western blotting.

Due to progresses in several genetic approaches, the development of BC diagnosis and treatment has been accelerated. Although the development and validation of molecular assays remained deficient for BC detection and preclinical decisions (Zakharchenko et al., 2011), progress in this regard is fundamentally required for the rapid management of BC.

METABOLOMICS AND BREAST CANCER MANAGEMENT

One of the recent promising research areas in treating BC is metabolomics, which focuses on the study of metabolites and their metabolic pathways, which are quite different from the normal cell pathways. Metabolism can be studied in two ways: targeted and untargeted. Metabolomics databases are used to interpret metabolomics data through various bioinformatics tools such as mass spectrometer (MS) combined with chromatography and nuclear magnetic resonance (NMR) through which metabolic fingerprints and profile of specific samples can be generated (Cheung et al., 2019).

Metabolomics data generated can be applied to hunt for novel molecular biomarkers involved in BC prognosis, to monitor their metastatic state, drug response, and in making therapeutic decisions for BC management. Metabolomics is emerging fast in precision medicine, by which a personalized treatment is designed for a specific patient according to the patient's molecular abnormalities represented by the metabolomics profile and fingerprints. Likewise, through pharmacometabolomics, drug response can be studied in patients by keeping their metabolic profile in view (Xu et al., 2012; Peng et al., 2015; Marshall and Powers 2017).

Recently, diet-related metabolites are extensively explored to relate with the risk of BC development and susceptibility (Playdon et al., 2017). This study represents various diet circulating metabolites can be robust and informative such as tocopherols (vitamin E), butter-related caprate, alcohol-related metabolites medium-chain SFA, fried food–related 2-hydroxyoctanoate, an odd-carbon MUFA, a hydroxy fatty acid, animal fat metabolites, and dessert-related g-CEHC can be associated with the risk of BC development in ER+ cases (Key et al., 2006; Playdon et al., 2017). After exploring metabolomics profiles and related pathways, it is suggested that the mechanism of developing BC through diet-related metabolites include alteration in various physiological processes such as the tumor suppression, immune function, and response to growth factors by breast cells, estrogen synthesis elevation in adipose tissues, and inflammation (Sczaniecka et al., 2012; Johnson et al., 2013). It is also observed by candidates' dietary biomarkers which drive that androgen-dependent and androgen-independent mechanisms may induce alcohol-related BC particularly in postmenopausal ER+ cases.

Overall, studying diet-related metabolites and exploring the metabolic profiles of BC patients can give significant insights for developing dietary guidance for breast cancer prevention. However, challenges still exist in upgrading the technology for integrating such big data including metabolomics with other omics datasets. Nonetheless, metabolomics can play a crucial role in breast cancer diagnosis, understanding the molecular mechanism, and time management.

RADIOMICS AND BREAST CANCER MANAGEMENT

Radiomics is an emerging field, and it provides quantitative and qualitative imaging biomarkers for the diagnosis, staging, distant-metastasis detection, therapeutic and prognostic prediction, and evaluation of therapeutic responses of BC. It is a method that uses data from clinical radiographic images through data-

characterization algorithms and interprets the information by advanced computational analyses. PET/CT and MRI scans are widely used to evaluate the BC's diagnosis, progression, and treatment success. Recent studies have proven that the combination of these techniques is more effective than individual ones. The clinical application use of PET/MRI/CT scans in detection of primary breast cancer is effective. Although PET, CT, and MRI scans have been used in the diagnosis of the primary breast cancer, however, their sensitivity and specificity differs depending upon the type of the breast cancer. The use of these technologies in different studies and their outcomes has been summarized by Ming et al. (2020). Conclusively, localized breast cancer can be better diagnosed with PET and MRI, while axillary and extra-axillary nodal metastases have been better diagnosed by combining PET/CT or PET/MRI. Additionally, PET/CT is superior in terms of monitoring local recurrence.

Radiogenomics is an emerging field of radiomics, which combines the information from clinical images and genomic databases using artificial intelligence for BC type determination, treatment plan, and outcome measurements. The integrated process of radiogenomics, crucial strategies, and statistical algorithms involved in current studies has been summarized by Shui et al. (2020). The application of radiogenomics in breast cancer, challenges, and future perspectives have been discussed in detail by Pinker et al. (2018). Although international guidelines, workflow, and standard procedures need standardization, this new field provides hope for atomization of the whole process.

Nanobiotechnology and Breast Cancer Management

Nanotechnology offers numerous approaches for imaging, monitoring, diagnosing, and delivering chemotherapeutic drugs to the tumor site. Nanoparticles aid in providing medications with improved efficacy, lower toxicity, and the ability to bypass biological barriers, resulting in improved anticancer activity (Jain et al., 2020). Medication-loaded nanoparticles, micelles, and liposomes are examples of nanomedicine that have unique properties that allow them to pass through biological membranes and deliver the encapsulated drug to the cells. Nanotechnology has several distinguishing features, including small size (nanometric), active and passive targeting, the capacity to connect several targeting moieties, controlled release, and site-specific targeting. Particle size, shape, and surface chemistry are all characteristics that influence the cellular uptake, biodistribution, and clearance mechanisms (He et al., 2010). For diverse types of BC treatment, many other nanoparticulate chemotherapeutic delivery platforms have been in clinical trials (Alyassin et al., 2020).

Recent advances in nanoparticles imply that they could be used to target drugs selectively in BC without damaging normal cells and tissues. Reduced toxicity, biocompatibility, ease of manufacturing, and high encapsulation efficiency are all properties of nanoparticles. Nanoparticles have the advantage of isolating the medicine or encapsulated molecules from exposure to an external environment, which protects the drug while also protecting nearby cells (McClements 2018). To target Engrailed-1 (EN1), which is overexpressed in TNBC, Sorolla et al. produced a docetaxel-PGMA-PAA-nanoparticle conjugated with the peptide (EN1-RGD-iPep) (Jain et al., 2020). The results showed that employing peptide-functionalized nanoparticles inhibited cell proliferation and increased apoptosis. In addition, the dose of DTX encapsulated in nanoparticles was lowered from 20 mg/kg to 2 mg/kg. In T11 and SUM149 mouse models, peptide-conjugated nanoparticles improved antitumor activity, reduced tumor volume, and improved bioavailability and pharmacokinetics (Selot et al., 2016; Sorolla et al., 2019). In a separate investigation, PTX-loaded PEG-maleimide-fractionalized PLGA nanoparticles were coupled with an antibody for enhanced therapeutic efficacy in TNBC against perlecan (maintains endothelial barrier function). This work demonstrated that nanoparticles could improve tumor drug delivery to TNBC by showing increased cellular uptake, enhanced cytotoxicity, and tumor size reduction in these carriers. Classic medications such as gemcitabine and bevacizumab are used to treat a variety of malignancies, including ovarian, prostate, and breast cancers. In phase II therapeutic trial, gemcitabine and PTX-loaded albumin-stabilized nanoparticles were functionalized with bevacizumab mAb to decrease tumor development in BC patients. The study's outcome was 6-month progression-free survival (PFS). In another clinical trial, albumin nanoparticles bound with rapamycin were used to treat recurrent breast cancer and showed therapeutic efficacy with a 5-year patient survival rate. Inhibition of the mTOR pathway was the primary mechanism for tumor growth regression (Khanna et al., 2019).

Role of Nanobiotechnology to Overcome Multidrug Resistance in Breast Cancer

Anticancer medications encapsulated in nanoparticles can target tumor cells actively or passively, improving the therapeutic efficacy at the target region. Chemotherapy medications' systemic toxicity can be reduced, and certain types of multidrug resistance can be avoided (Yuan et al., 2016). Passive targeting occurs when nanoparticles travel through holes in leaky blood arteries and are held by the aberrant draining lymphatic system, resulting in the EPR effect (increased permeability and retention). Passive targeting can occur when positively charged nanoparticles electrostatically interact with the negatively charged sialic acid and phospholipids on the surface of tumor-associated endothelial cells (Palakurthi et al., 2012). Active biomolecules such as nucleic acids, peptides, sugars, and antibodies can modify nanoparticles to bind to cancer cells actively (Yuan et al., 2016; Sorolla et al., 2019). Ideally, high-affinity nanoparticles combine specifically with molecules such as carbohydrates, proteins, folate, transferrin, aptamers, or lipids. Nanoparticle delivery minimizes damage to non-cancer cells because these are overexpressed on the surface of cancer cells. Active targeting is used to recognize cells precisely using functional biomolecule interactions, improve drug endocytosis by the cell, reduce

cytotoxicity in non-cancer cells, raise drug concentration, and leverage the EPR effect (Ali et al., 2021). Once the cell is discharged into the cytoplasm, nanoparticles are taken in by the cell via endocytosis, frequently bypassing and avoiding old ABC-transporters responsible for lethal drug efflux (Choudhury et al., 2019). Clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis, and other endocytosis are the four primary mechanisms of endocytosis (Yuan et al., 2016). Apart from receptor-independent endocytosis, clathrin-mediated endocytosis is the best researched mechanism involved in receptor-mediated nanoparticle uptake. The transferrin, low-density lipoprotein (LDL) receptor, and epidermal growth factor receptors (EGFR), particularly the human epidermal growth factor receptor 2 (HER2), are all significant receptors (Patra and Turner 2014; Cerqueira et al., 2015). Nanoparticles that enter cells by clathrin-mediated endocytosis end up in lysosomes, where the acidic environment degrades them to release medicines (Malinovskaya et al., 2017). After nanoparticles bind to the cell membrane, caveolae-mediated endocytosis produces cytosolic caveolar vesicles. Folic acid, albumin, and cholesterol are ligands linked to caveolae-mediated endocytosis. Another nonselective endocytosis mechanism, macropinocytosis, is based on action-driven membrane protrusions that fuse with and separate from the plasma membrane to produce macropinosomes. In addition, nanoparticles encapsulating P-gp inhibitors and anti-cancer medicines can be employed to circumvent P-gp mediation MDR. Wong and colleagues mixed doxorubicin and elacridar, a P-gp inhibitor, in polymer-lipid hybrid nanoparticles. According to the findings, the simultaneous delivery of the two medications improved the treatment of multidrug-resistant breast cancer *in vitro* (Cerqueira et al., 2015; Yuan et al., 2016; McClements 2018; Sorolla et al., 2019).

Precision Medicine

The recent developments in the "OMICS" sciences have uncovered many cellular mechanisms. This has led to the foundation of a new field of study known as precision or personalized medicine. It has been an established fact now that not all the cancer patients respond to the same treatment regime equally. The difference in treatment responses is subjected to the variation in the genome that each individual carries. These variations lead to the variations in responses toward the drugs. Precision medicine is changing the healthcare pattern by linking individual genetic information to drug applications, thus changing the conventional practice of medicine.

Traditional Standards

The conventional standard where the treatment strategy was "one-dose-fits-all" has been ineffectual as it incurs all the risks of the following drug toxicities and treatment failures. The drug inefficacy has been observed in several patients for different diseases. The variation in failures is 38–75%, where patients have no effect of the drug (**Figure 2**). In cancer drugs, the response rate is as low as 25% (Spear et al., 2001).

The adverse reactions of drugs as a treatment consequence are another problem. In the United States, 16% have shown adverse reactions to drugs. Meta-analysis has revealed that around 6.7% of all the patients admitted in the hospitals of the United States are associated with adverse drug reactions with more than 100,000 death reports annually (Lazarou et al., 1998; Spear et al., 2001). This makes precision medicine an advanced approach to the future medicine.

Precision Medicine and Breast Cancer

Breast cancer is the primary cause of cancer in women globally (Sung et al., 2021). Recently, molecular investigations have shown that it is a combination of several diseases with various biological behaviors rather a single disease. Thus, precision medicine is the best choice in such circumstances. This new approach of oncology is utilized at different levels of breast cancer management, which includes treatment efficacy prediction, prognosis, and development of new treatments through new types of clinical trials. These trials would not only include the breast cancer targeting but also characterization of tumor genetics *via* advanced molecular genomics techniques such as next-generation sequencing. The aim of the precision medicine is to customize treatment according to the disease specificity for a given patient.

Preventive Strategy

Precision medicine digs into the personal genetic and protein profiles to improve the healthcare at a more personalized level, with the help of the recently emerging "-omic" technologies that include genomics, proteomics, metabolomics, and pharmacogenomics (Tebani et al., 2016; Ahmed 2020). These techniques have enabled us to forecast the disease or its presence before the clinical symptom's appearance. It enables us to act on the disease through an early intervention that can save lives in many cases. The analysis of the molecular characteristics of primary stage breast cancer using next-generation sequencing has led to the portrayal of the genomic background of the disease (Stephens et al., 2012). Such information is key for designing preventive schemes. For example, females carrying genetic mutations in the genes *BRCA1* or *BRCA2* have a greater chance of developing breast cancer (Riis 2021). Similarly, mutations of *TP53* and *PIK3CA* are the frequent genomic alteration in all intrinsic subtypes (Stephens et al., 2012). Other mutations are less frequent, but could be clinically relevant, that includes mutations and deletions in *PTEN*, *RB1,* and *AKT1* mutations. Sequencing information has identified mutations in other genes of interest that might be clinically relevant in breast cancer such as *KRAS*, *NF2*, *SKT11*, *APC*, and *AKT2*. A precise test of these breast cancer-associated genes can guide examination and preventive treatment based on the objective risk measurement such as the increased frequency of prophylactic surgery, chemoprevention, and mammography (Cui et al., 2014).

Prediction Strategy

Precision medicine facilitates physicians to opt for therapies which are best suitable for the patients (**Figure 3**). This allows avoiding the adverse drug reactions. The molecular diagnostic

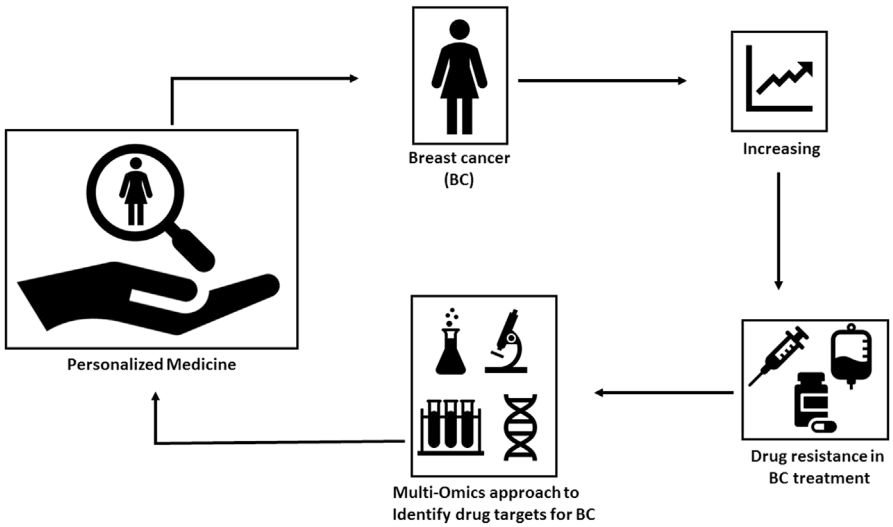

FIGURE 1 | Multiomics approaches and breast cancer management.

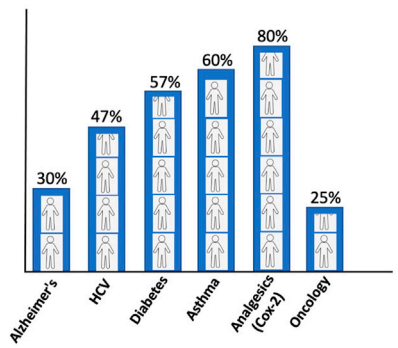

FIGURE 2 | Patients' response rate to major drugs against different diseases (Spear et al., 2001).

devices used for the detection of predictive biomarkers provide vital information regarding the genetics of the patients who will benefit from a defined therapy. This type of subtyping is also applied in early breast cancer for the determination of the modality and decision for antitumor agents that best suit the patient (Sabatier et al., 2014). There are several multigene assays present that estimate the associated risks (**Table 2**). For example, MammaPrint (Agendia, Netherlands) utilize samples to examine 1,391 genes *via* microarray assays, and the results of 70 gene expression profile are used to assess the risk and classify patients in high to low risk for relapse (Van't Veer et al., 2002; Van De Vijver et al., 2002). Oncotypes DX (Genomic Health, United States) is another generally used multigene assay. It uses a 21-gene signature (includes five reference genes) to conclude whether the patient with a certain breast cancer type would benefit from chemotherapy (Hornberger et al., 2005; McVeigh et al., 2014; Dafni et al., 2019). PMA50-based Prosigna (NanoString Technologies) analyzes a signature of 50 genes to assess the risk of recurrence (ROR) (Lænkholm et al., 2018). Similarly, EndoPredict (Myriad Genetics) is another breast cancer prognostic test. It does RNA expression analysis of eight target genes along with three normalization genes and a control gene. This information is used to predict ROR in patients with breast cancer at 10 years (Sestak et al., 2019). These diagnostic tests lead to the patient's classification into subgroups that help physicians to make a treatment decision whether hormone therapy alone would be sufficient or may require chemotherapy. These assays are currently employed in practice guidelines and been utilized in the clinic.

Targeted Strategy

In recent times, molecular medicine has advanced, which is the key to precision medicine. In breast cancer, many potential druggable mutations have been determined. However, there is comparatively less proof to support the usage of matched molecular targeted agents in breast cancer. Mutation in *PIK3CA* occurs in approximately 25% of breast cancer, and it is reported as a driver of this disease (Cancer Genome Atlas Network 2012). However, in the early clinical trials, the use of the non-selective *PI3K* inhibitors led to modest response rates (4%) while administered as a monotherapy (Mayer et al., 2014), whereas the second-generation (α-selective) *PI3K* inhibitor produces improved inhibition *in vivo* in animal models and is more specific (Fritsch et al., 2014). Although initial results with these new inhibitors showed partial responses of about 6% in patients with *PIK3CA* (mutant) breast cancer, no responses had been detected in patients with *PIK3CAI* (wild-type) tumors (Janku et al., 2015). Therefore, it is important to

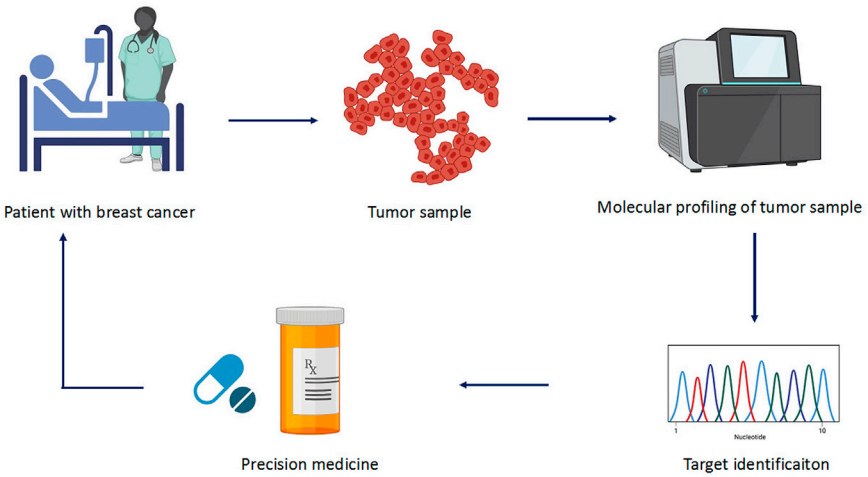

FIGURE 3 | Different steps involved in precision medicine. The sample is collected from patients who are subjected to molecular profiling or high-throughput sequencing. The collected data are analyzed for mutation and target genes involved in the cancer. Based on the identified target, precise drug is selected to treat the cancer.

TABLE 2 | Diagnostic devices used for breast cancer multigene assays.

Assay	Sample	Number of genes	Analysis	Company
MammaPrint	Fresh/frozen paraffin-embedded (Fresh/frozen)	70	Microarray	Agendia (Netherlands)
Oncotype DX	Fresh/frozen paraffin-embedded	21	qRT-PCR	Genomic Health (US)
PAM50	Fresh/frozen paraffin-embedded (Fresh/frozen)	55	Microarray/qRT-PCR	ARUP Laboratories (US)
EndoPredict	Fresh/frozen paraffin-embedded	11	qRT-PCR	Myriad (US)

design drugs which can hit the target with maximum specificity and bioactivity. The presence of mutation (two or more) in cancer-associated genes has been linked with resistance to targeted therapies *in vitro* and in clinical trials (Arnedos et al., 2015). It has been reported in the breast cancer that 67% of samples analyzed carry two or more genomic mutations. There are many such observed issues in breast cancer, for example, the co-existence of mutations of *PIK3CA* and amplification of *ERBB2* have been linked with resistance to *HER2*-targeting drugs lapatinib and trastuzumab (Fontanella et al., 2014), which provides the rationale of combining *HER2* and *PI3K* inhibitors in different clinical trials. However, such resistance toward anti-*HER2* therapy with the presence of *PIK3CA* mutations was not being observed when double blockage for *HER2* was attained via the usage of two monoclonal antibodies (Baselga et al., 2014). Cancer itself is also subjected to evolve through genetic diversification in a complex pattern. Therapeutic interventions might reduce or control cancer, but it might also stimulate the development of resistant variants (Wang et al., 2014; Eirew et al., 2015), such as tumor adaption via initiation of alternative protein networks which will dodge targeted inhibition. Such a mechanism has been reported in patients who were treated with *mTOR* inhibitors, whereas a feedback mechanism by *mTORC2* was observed which resulted in *AKT* activation *via* growth factor receptor phosphorylation (*IGF-1R*) (Garay et al., 2015), which provide the foundation to use these inhibitors with *IGF-1R* (Atzori et al., 2011; Chen et al., 2013) or PI3K inhibitors. Thus, precise and timely detection of drug targets or any associated resistance is vital for therapy optimization which can be achieved *via* precision medicine.

CONCLUSION

Breast cancer is a very complex and heterogeneous illness with unique molecular and morphological features. Recent advancements in the omics technology have allowed a more precise approach toward the breast cancer classification by understanding the underlying molecular mechanisms. However, there is need for the integration of multiomics approaches which could take omics data from an individual patient and compare it with the databases to guide the strategy for personalized therapy. For management of the breast cancers, the treatment decisions not only depend on the assessment of prognosis factors but also on the evaluation of pathological and clinical factors. An integrated data approach of

these multiple factors of breast cancer through multiomics can provide significant insight and hope for making therapeutic decisions. Such personalized therapies will avoid conventional therapeutics where one medicine fits all. It will not only facilitate the patient's treatment time but also their long-term sufferings as well.

AUTHOR CONTRIBUTIONS

SM searched the topic, wrote the manuscript, and involved in discussion and drafting of the manuscript. MF, HI, SF, SY and MI participated in writing the manuscript. MA was involved in manuscript writing, discussion and drafting of the manuscript.

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BRIEF RESEARCH REPORT
published: 17 May 2022
doi: 10.3389/fmolb.2022.818493

Mir-4728 is a Valuable Biomarker for Diagnostic and Prognostic Assessment of HER2-Positive Breast Cancer

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Breast cancer remains one of the most common malignancies in female cancer patients. The rapid and accurate diagnosis of human epidermal growth factor receptor 2 (HER2) status is indispensable for breast cancer patients. The pre-miR-4728 (mir-4728) is encoded within an intron of the HER2 gene. We showed here that mir-4728 was the most significantly upregulated pre-miRNA in HER2-positive breast cancer patients (fold-change: 4.37), and it could serve as a strong diagnostic factor for the HER2 status in breast cancer patients ($p < 0.0001$). Moreover, mir-4728 was positively correlated with tumor recurrence and appeared to be a critical independent risk factor of tumor recurrence in patients with high tumor burden (HR: 7.558, 95% CI:1.842-31.006, $p = 0.005$). Remarkably, HER2-positive patients with higher mir-4728 expression levels had better drug responses to targeted therapies. Furthermore, estrogen receptor (ESR), the predictive marker for endocrine therapies, was found to be the direct target of miR-4728-3p. Taken together, our results supported the potential role of mir-4728 in the diagnosis of HER2 status and the prognostic assessment of HER2-positive patients in response to targeted therapies.

Keywords: breast cancer, HER2, mir-4728, early diagnosis, ESR

OPEN ACCESS

Edited by:
Dongqing Wei,
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Reviewed by:
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Specialty section:
This article was submitted to Molecular Diagnostics and Therapeutics, a section of the journal Frontiers in Molecular Biosciences

Received: *19 November 2021*
Accepted: *28 March 2022*
Published: *17 May 2022*

Citation:
Rui T, Xiang A, Guo J, Tang N, Lin X, Jin X, Liu J and Zhang X (2022) Mir-4728 is a Valuable Biomarker for Diagnostic and Prognostic Assessment of HER2-Positive Breast Cancer. Front. Mol. Biosci. 9:818493. doi: 10.3389/fmolb.2022.818493

INTRODUCTION

Although multimodality treatment has contributed to improving breast cancer prognosis, breast cancer remains one of the major causes of cancer mortality in female cancer patients (DeSantis et al., 2019; Siegel et al., 2019). Human epidermal growth factor receptor 2 (HER2)-positive breast cancer is a particular class of breast cancer, which accounts for 25% of all breast cancers and has a poor prognosis. Fortunately, comprehensive targeted therapy can significantly prolong HER2-positive patient survival (Ross and Gray, 2003; Escrivá-de-Romaní et al., 2018). Thus, an accurate and rapid diagnosis of the HER2 status is essential for the timely treatment of breast cancer patients. Currently,

Abbreviations: AUC, area under the curve; ESR, estrogen receptor; HER2, human epidermal growth factor receptor 2; mir-4728, pre-miR-4728; mirna, pre-miRNA; PGR, progestogen receptor; TCGA, The Cancer Genome Atlas; 3'UTR, 3' untranslated region.

the technique of HER2 diagnosis depends on immunohistochemistry (IHC) (Pauletti et al., 2000). The patients whose IHC score is 3+ could be diagnosed with a HER2-positive status, while the patients with an IHC score of 2+ must be further detected with fluorescent *in situ* hybridization (FISH) assay (Carlson et al., 2006), which is costly and not widely available. Only a few qualified institutions could provide such diagnostic services. Therefore, it is necessary to explore a more straightforward method to examine the HER2 status.

The novel mir-4728 (pre-miR-4728) is encoded within an intron of the HER2 (ErbB2) gene (Ch17:39726495-39726561) (Persson et al., 2011). A co-amplification or co-expression might exist between mir-4728 and HER2. Thus, we hypothesized that the expression of mir-4728 could reflect the HER2 status of breast cancer. The *in vitro* functions of mir-4728 in promoting breast cancer malignancy have been reported (Li et al., 2015; Floros et al., 2018). However, the correlation between mir-4728 expression and HER2 status in breast cancer remains to be explored, especially the potential application of mir-4728 in clinical diagnosis.

The status of estrogen receptor (ESR) or progestogen receptor (PGR) also guides the treatment of breast cancer patients in clinical applications. After combining systematic endocrine therapy, ESR and PGR-positive breast cancer patients can receive a favorable prognosis (Hammond et al., 2011; Jorns, 2019). As three key markers for breast cancer, the association between the status of HER2 and ESR or PGR warrants further attention and investigation. In this study, we focused on the potential value of mir-4728 in the diagnosis of HER2 status and prognostic evaluation for breast cancer. The correlation between mir-4728 and ESR or PGR was also established.

MATERIALS AND METHODS

Data Acquisition and Analysis

As described previously (Rui et al., 2020a), the miRNA-sequencing data, mRNA-sequencing, and the matched clinical data of breast cancer samples and non-tumor samples were downloaded from The Cancer Genome Atlas (TCGA) database (https://cancergenome.nih.gov/). The HER2 status of breast cancer patients has been diagnosed in TCGA database. By using the "edgR" R software package, the differentially expressed miRNAs in HER2-positive breast cancer patients were normalized, screened, and compared with HER2-negative patients. Log_2 fold change > 1 and FDR (false discovery rate) < 0.05 were set as the satisfying criteria. The complete clinicopathological information of the patients was also acquired from TCGA database. Clinical characteristics for the patients from TCGA are shown in **Supplementary Table S1**.

Cell Culture

The breast cancer cell lines, MCF-7 and ZR-75-1, were purchased from the Cell Bank of the Chinese Academy of Sciences. The Roswell Park Memorial Institute 1640 Medium (GIBCO, United States) with β-estradiol (1 nM) was for ZR-75-1. The Minimum Essential Medium (GIBCO, United States) was for MEC-7. All media were supplemented with 10% FBS (BioIND, China). The cells were cultured in a humidified incubator with the condition of 5% CO_2 at 37°C.

Cell Transfection

For evaluating the targets of miR-4728-3p and miR-4728-5p in breast cancer, miR-4728-3p mimic oligonucleotides, miR-4728-5p mimic oligonucleotides, and their controls (RiboBio, China) were synthesized. The efficient RNA transfection reagent RiboFECT (RiboBio, China) was used to transfect the RNA oligonucleotides (250 nM) according to the manufacturer's instructions.

Dual-Luciferase Reporter Assay

As described previously (Rui et al., 2021), the pmirGLO vector (Repobio, China) was designed to assess the inhibitory effect of miRNAs by measuring the luciferase activity. The sequences of wild-type or the mutant-type 3' untranslated regions (3'UTRs) of the ESR were cloned into the pmirGLO vector. Then, the 293T cells were co-transfected with the constructed vectors and miR-4728-3p mimics or control mimics by using lipofectamine 3000 (Thermo Fisher Scientific, United States). After 48 h, a dual-luciferase activity was detected with the Dual-Luciferase Reporter assay kit (Vazyme, China), according to the manufacturer's instructions.

Western Blot Analysis

As described previously (Rui et al., 2020b), the total proteins extracted from the MFC-7 and ZR-75-1 were quantified and boiled. Then, the total proteins were electrophoresed with 10% SDS-PAGE gel (FD341-100, Fudebio, China) and transferred onto equilibrated polyvinylidene-difluoride membranes. The membranes were blocked with 5% BSA for 1 hour at room temperature and incubated with anti-ESRα (A0296, Abclonal, China), anti-PGR (A0321, Abclonal, China), and anti-GAPDH (A19056, Abclonal, China) overnight at 4°C. Before being detected by an enhanced chemiluminescence system (Biotanon, China) with FDbio-FemtoEcl (FD8380, Fudebio, China), the membranes were incubated with the secondary anti-rabbit IgG HRP-linked antibody.

Statistical Analysis

The quantitative data were expressed as the mean ± standard deviation (SD) or median ± interquartile range (IQR). The Student's *t*-test or Mann–Whitney test was performed to compare the quantitative variables between the two groups. The optimal cutoff value of mir-4728 for diagnosing the HER2 status of breast cancer was detected with the ROC curve, by calculating the best Youden index, considering both sensitivity and specificity. A one-way ANOVA followed by a post hoc Bonferroni test was used to analyze more than two groups. Categorical measures were compared with the chi-square test or Fisher exact test. The Kaplan–Meier survival curves were assessed with the log-rank test. The independent risk factors of breast cancer recurrence were screened with the multivariate Cox proportional hazards regression analysis. A statistical analysis was performed with the SPSS Software (Version 19.0),

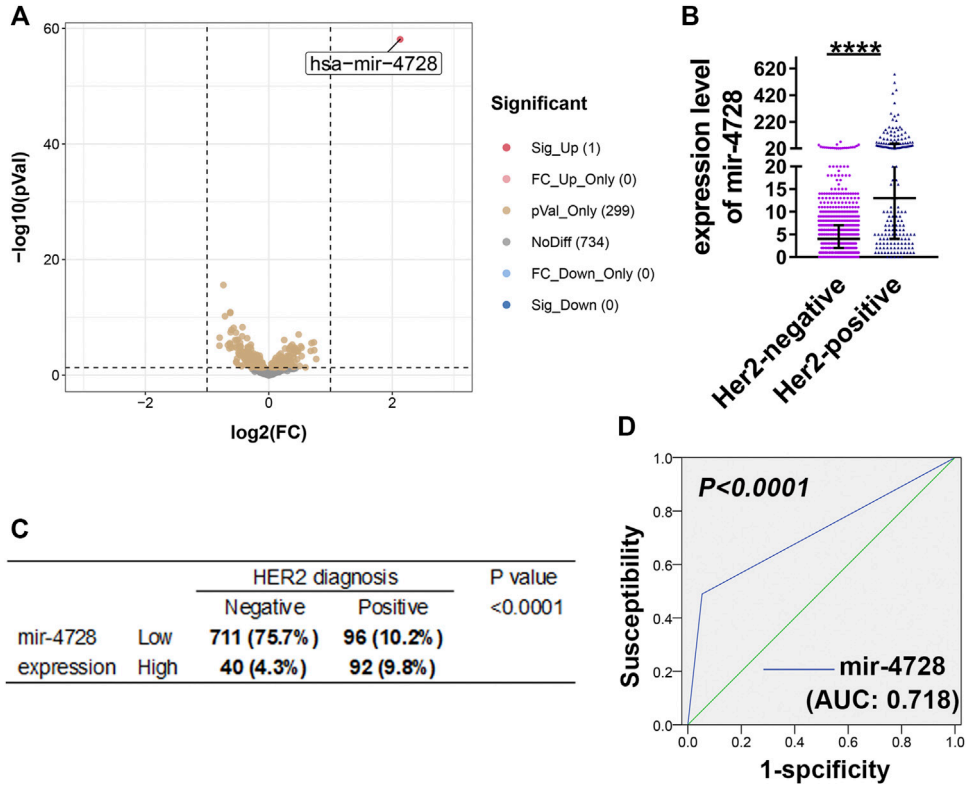


FIGURE 1 | Mir-4728 acts as a key marker in the diagnosis of HER2 status. **(A)** Volcano plot depicts the differentially expressed mirnas between breast cancer patients with HER2-positive status and HER2-negative status. **(B)** Mir-4728 is significantly upregulated in HER-2 positive patients in comparison with HER2-negative patients. **(C)** Correlation analysis between the mir-4728 expression and HER-2 status diagnosis in breast cancer patients. **(D)** The area under the receiver operating characteristic (AUROC) shows that mir-4728 significantly predicts the HER-2 status diagnosis in breast cancer patients.

and the p-value < 0.05 was set as the significance level. "*", "**", "***" and "****" indicated p-value < 0.05, 0.01, 0.001, and 0.0001, respectively.

RESULTS

Mir-4728 Is a Strong Diagnostic Factor for the Human Epidermal Growth Factor Receptor 2 Status of Breast Cancer Patients

Based on the miRNA sequencing results and HER expression levels from TCGA, we enrolled the data of 939 breast cancer samples. Then, we divided the patients into HER2-positive and HER2-negative groups according to the HER2 status diagnosis and screened the differentially expressed miRNAs (at the criteria of $\log_2$ fold-change > 1 and FDR > 0.05). We found that mir-4728 was a unique miRNA with the most significant and highest expression in HER2-positive breast cancer patients (**Figure 1A**; **Supplementary Material S1**), with a fold-change up to 4.37 (**Figure 1B**). The results showed that the HER2-positive patients expressed significantly higher levels of mir-4728, which indicated the potential links between the mir-4728 expression level and the HER2-positive status. Thus, using the chi-square test, we confirmed that the expression of mir-4728 was significantly correlated with HER2 status (**Figure 1C**). Low mir-4728 levels were particularly associated with a HER2-negative status. The ROC curve further showed that mir-4728 expression levels predicted the HER2 status in breast cancer patients. The area under the curve (AUC) was 0.718 (p < 0.0001) (**Figure 1D**). The values of specificity and sensitivity were 94.9 and 66.2%, respectively. The positive predictive value and negative predictive value were 82.5 and 88.7%, respectively. The results suggested that mir-4728 might be a valuable marker for the prediction of HER2 status in breast cancer patients.

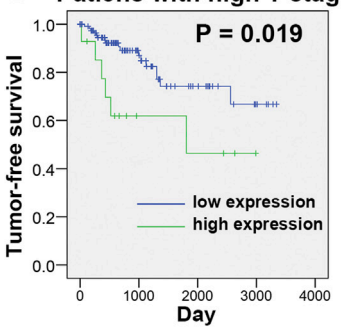
Mir-4728 Predicts the Early Recurrence of Breast Cancer and Guides the Therapy for Breast Cancer

We further evaluated the utility of mir-4728 in the prognosis assessment of breast cancer. Using the chi-square test, we found that in breast cancer patients at the high T stage, the mir-4728 expression was positively correlated with disease recurrence (p = 0.043) but not with tumor survival (**Figure 2A**). The Kaplan-Meier analysis with log-rank test also confirmed that higher expression of mir-4728 predicted lower tumor-free survival, only in the high T stage patients (p = 0.019) (**Figure 2B**;

A

	Characteristics	mir-4728 expression		p
		Low	High	
Low T-stage	Overall survival			
(I+II)	Alive	613	107	0.531
	Dead	65	9	
High T-stage	Overall survival			
(III+IV)	Alive	108	10	0.104
	Dead	21	5	
Low T-stage	Tumor recurrence			
(I+II)	No	621	107	0.815
	Yes	57	9	
High T-stage	Tumor recurrence			
(III+IV)	No	**106 (73.6%)**	**9 (6.3%)**	**0.043**
	Yes	**23 (16.0%)**	**6 (4.2%)**	

B



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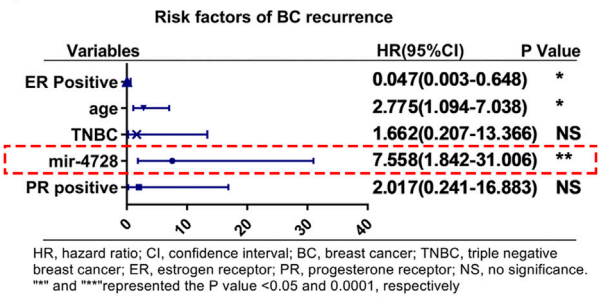

HR, hazard ratio; CI, confidence interval; BC, breast cancer; TNBC, triple negative breast cancer; ER, estrogen receptor; PR, progesterone receptor; NS, no significance. "*" and "**"represented the P value <0.05 and 0.0001, respectively

D

			Therapy response		P value
			CR	PR+PD	
Chemotherapy	mir-4728 expression	Low	61	5	0.578
		High	17	0	
Hormone therapy	mir-4728 expression	Low	13	1	0.242
		High	1	1	
Chemotherapy+ Hormone therapy	mir-4728 expression	Low	66	7	0.361
		High	3	1	
Chemotherapy+ Hormone therapy+ Targeted therapy	mir-4728 expression	Low	**0**	**2**	**0.018**
		High	**9**	**0**	

FIGURE 2 | Potential clinical implication of mir-4728 in breast cancer patients. **(A)** Correlation analysis between the mir-4728 expression and tumor recurrence in breast cancer patients at the high T and low T stages. **(B)** Log-rank statistics show that high mir-4728 expression predicts early tumor recurrence in the patients with high T stage. **(C)** Independent risk factors of tumor recurrence in patients at high T stage. **(D)** Correlation analysis between the mir-4728 expression and therapy response in the patients with different therapeutic strategies.

A

		mir-4728 expression		
		low	high	P value
Her2 negative	PGR Negative	222	15	0.420
	PGR Positive	485	25	
Her2 positive	PGR Negative	**27**	**44**	**0.005**
	PGR Positive	**69**	**48**	
Total	PGR Negative	249	59	0.002
	PGR Positive	554	73	
Her2 negative	ESR Negative	161	9	0.979
	ESR Positive	549	31	
Her2 positive	ESR Negative	**14**	**28**	**0.008**
	ESR Positive	**82**	**63**	
Total	ESR Negative	175	37	0.097
	ESR Positive	631	94	

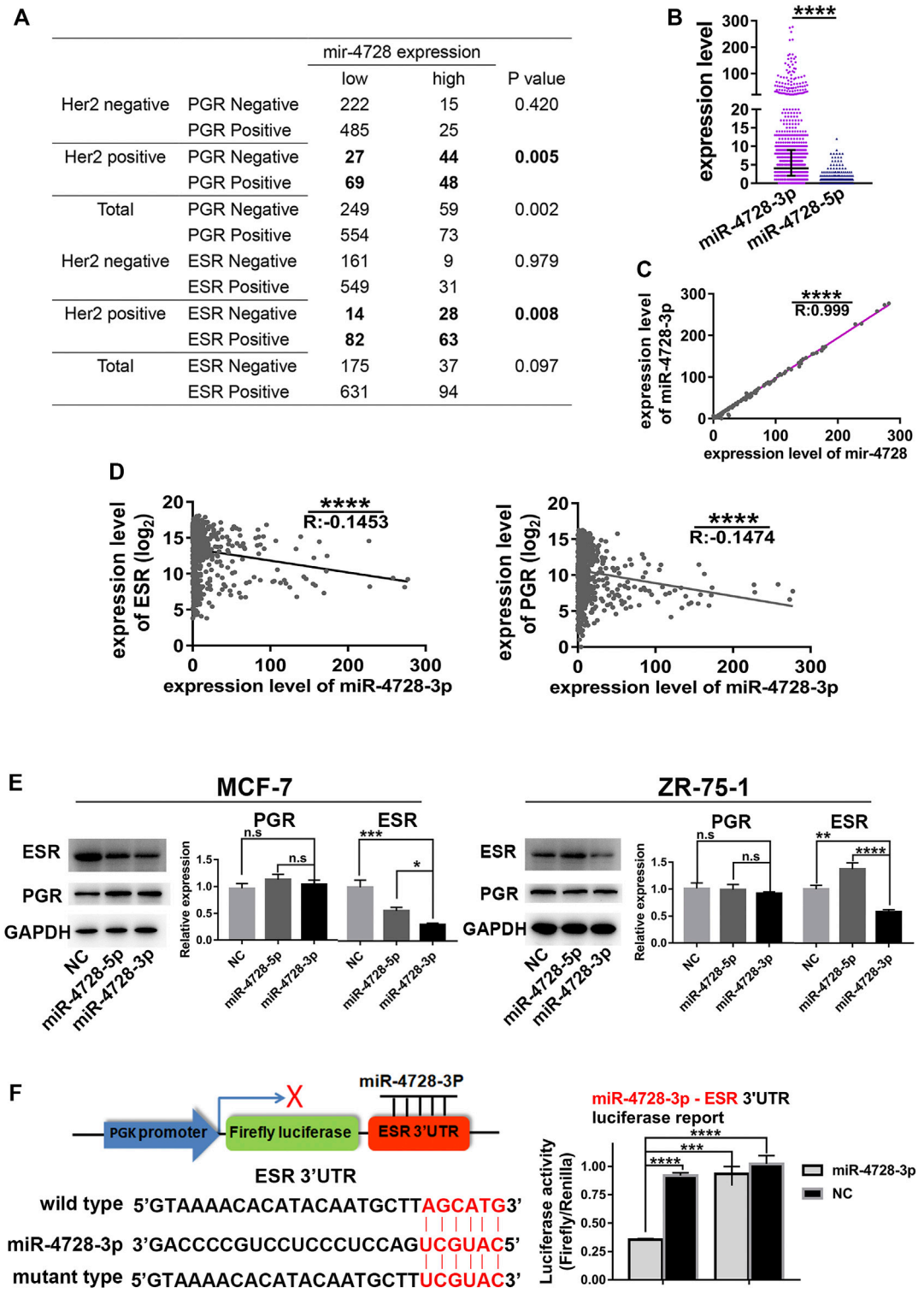

FIGURE 3 | MiR-4728-3p, spliced from mir-4728, directly targets the expression of ESR. **(A)** Correlation analysis between the expression of mir-4728 and ESR or PGR in the patients with different HER2 statuses. **(B)** The expression of miR-4728-3p and miR-4728-5p in breast cancer. **(C)** Linear correlation analysis between the expression of mir-4728 and miR-4728-3p. **(D)** Linear correlation analysis between the expression of miR-4728-3p and ESR (left) or PGR (right). **(E)** Western blot detects the change of ESR and PGR expression under the control of miR-4728-3p, miR-4728-5p, or the controls. **(F)** Dual-Luciferase assay shows the relative luciferase activities (Firefly/Renilla) among the 293T cells co-transfected with miR-4728-3p or controls and wild-type or mutant-type Dual-Luciferase reporters.

Supplementary Figure S1). A multivariate Cox proportional-hazards model showed that mir-4728 was the most important independent risk factor of tumor recurrence in patients at the high T stage (HR: 7.558, 95% CI:1.842-31.006, $p = 0.005$) (**Figure 2C**). These results suggested that mir-4728 had a significant predictive value in the recurrence of high tumor burden in breast cancer.

Given the intensive correlation between the expression of mir-4728 and HER2 status in breast cancer patients, we speculated a potential role of mir-4728 in guiding drug therapy for the patients. Following the designed therapeutic regimens, we stratified the patients into four treatment groups (chemotherapy; hormone therapy (endocrine therapy); chemotherapy combined with hormone therapy; and hormone therapy combined with targeted therapy). The results indicated that patients with targeted therapy who expressed higher mir-4728 responded better (all nine patients were in complete remission) ($p = 0.018$) (**Figure 2D**). This indicated that mir-4728 could predict the effect of targeted therapy and guide drug therapy against breast cancer. However, because of the small number of patients enrolled, the results need to be further verified with much more extensive clinical samples.

Estrogen Receptor But Not Progestogen Receptor is the Direct Target of miR-4728-3p

Interestingly, data from TCGA indicated that the expression of mir-4728 was negatively associated with ESR-positive and PGR-positive diagnoses, especially in HER2-positive patients (**Figure 3A**). We suspected that in HER2-positive patients, one of the mature miRNAs, miR-4728-3p or miR-4728-5p, spliced from the high expression of mir-4728, might inhibit the expression of ESR or PGR. First, we must determine the mature functional form of mir-4728. We found that the expression of miR-4728-3p was significantly upregulated in breast cancer than that of miR-4728-5p (fold-change: 30.33) (**Figure 3B**). A linear regression analysis also showed that there was an extremely significant correlation between miR-4728-3p and mir-4728 (R: 0.999, $p < 0.0001$) (**Figure 3C**). All the results showed that miR-4728-3p was the mature functional miRNA spliced from mir-4728.

Next, we further attempted to detect whether miR-4728-3p could inhibit the expression of ESR or PGR. The mRNA levels of ESR and PGR from TCGA database were acquired. A linear regression analysis showed that miR-4728-3p was significantly negatively correlated with both ESR and PGR levels, indicating that miR-4728-3p might inhibit the expression of ESR and PGR (**Figure 3D**). The experiment was also performed to confirm that ESR and PGR expression changes under the control of miR-4728-3p. First, the ESR and PGR-positive breast cancer cell lines MCF-7 and ZR-75-1, were transfected with miR-4728-3p mimics, miR-4728-5p mimics, or their controls, respectively. A Western blot showed that miR-4728-3p significantly inhibited the expression of ESR, which did not occur with the miR-4728-5p and control counterpart (**Figure 3E**). However, the PGR expression was not markedly changed by miR-4728-3p, indicating that PGR was not the target of miR-4728-3p (**Figure 3E**). To further confirm that ESR was the direct target of miR-4728-3p in breast cancer, we predicted the potential target sites of 3'UTR of ESR from the miRWalk database (http://mirwalk.umm.uni-heidelberg.de) (Sticht et al., 2018). The wild-type and mutant luciferase reporter vectors of ESR-3'UTR were constructed. Then, the miR-4728-3p mimics or control mimics and wild-type 3'UTR vector or mutant 3'UTR vector were co-transfected into 293T cells, for the Dual-Luciferase Reporter assay. The results showed that the activity of luciferase was significantly inhibited in the miR-4728-3p-wild-type vector group (**Figure 3F**). Thus, miR-4728-3p, the functional form of mir-4728, could directly target the expression of ESR.

DISCUSSION

The status of HER2, ESR, PGR, and Ki67 guide the classification of the four molecular subtypes of breast cancer (Perou et al., 2000; Sørlie et al., 2001). Meanwhile, HER2 status basically guides the whole process of breast cancer treatment. Not only does targeted therapy (trastuzumab, pertuzumab, and TDM-1) depend on the diagnosis of HER2 status, but the HER2 status also affects the chemotherapy strategy for breast cancer (Gradishar et al., 2020). Therefore, it is necessary to diagnose the HER-2 status before treating breast cancer. The diagnosis of HER2 status relies on the IHC and FISH analyses. However, it is equivocal by FISH when HER2: CEP17 (centromere) is < 2.0, but HER2 signals are ≥ 4 but < 6 (Agersborg et al., 2018). In this study, we aim to discover the ideal biomarker for easily and accurately diagnosing the HER2 status of breast cancer. MiRNAs or pre-miRNAs, as one kind of small non-coding RNAs, are the emerging biomarker candidates in multiple cancers (Bjorkman and Taylor, 2019). Persson et al. (2011) showed that mir-4728 is from the intron of the HER2 gene, suggesting that the expression of mi-4728 may reflect the HER2 status. Li et al. (2015) also confirmed that miR-4728-3p is upregulated in HER2-positive breast cancer patients. However, the exact diagnostic value of mir-4728 to HER2 status remains unclear. We showed in this study that mir-4728 was a unique miRNA with the most significant high expression in HER2-positive patients, compared with the HER2-negative patients. The ROC curve indicated that mir-4728 could well predict the HER2 status of breast cancer, with the specificity being as high as 94.7%. Our results strongly suggested that mir-4728 could serve as a biomarker for the diagnosis of HER2 status.

Clinically, compared with the other two molecular subtypes (luminal A and luminal B), patients with a HER2-positive status have poorer survival and a higher rate of distant metastasis (Kennecke et al., 2010; Godoy-Ortiz et al., 2019). In view of the high correlation between mir-4728 and HER2 status, we speculated that mir-4728 could have a predictive role in the prognosis of breast cancer. Our results confirmed that mir-4728 predicted the early recurrence of breast cancer patients with a high tumor burden. Meanwhile, mir-4728 was the independent risk factor for breast cancer recurrence.

High tumor burden breast cancer patients who had high levels of mir-4728 had a remarkably high recurrence risk (HR: 7.558, 95% CI: 1.842-31.006). The breakthrough of targeted therapy can provide more therapeutic options for HER2-positive breast cancer patients. Trastuzumab, pertuzumab, and TDM-1 have offered a better prognosis for HER2-positive patients (Loibl and Gianni, 2017). Interestingly, our data suggested that HER2-positive patients with a high mir-4728 expression could respond better to targeted therapy. However, the results based on TCGA database are considered as incomplete and preliminary. Thus, a clinical trial to confirm that mir-4728 could guide the targeted therapy of HER2-positive breast cancer is warranted.

ESR and PGR-positive patients who receive endocrine therapy can have a better prognosis (Cheung et al., 2000; Early Breast Cancer Trialists' Collaborative Group (EBCTCG), 2005). So far, there are few studies on the association between mir-4728 and ESR or PSR status. Our analyses indicated that there were significant negative correlations between mir-4728 and ESR or PGR status. However, when we transfected the miR-4728-3p or miR-4728-5p into breast cancer cell lines, the expression of PGR was not inhibited, suggesting that PGR was not the direct target of mir-4728. The indirect connection and potential regulatory mechanisms between mir-4728 and PGR status may deserve further exploration. Previously, Newie et al. (2014) showed another seed site of ESR 3'UTR that miR-4728-3p binds to. In this study, we confirmed that miR-4728-3p was the functional miRNA spliced from mir-4728. Through Western blotting and dual-luciferase reporter assay, we demonstrated that ESR was the direct target of miR-4728-3p. These results suggest that the poor prognosis of HER-positive status in breast cancer may be partly due to the suppression of ESR by the co-expression of mir-4728.

There have been limitations in this study. The results came from the sequencing data of the TCGA database. Therefore, it should be further verified in clinical samples. Also, this study showed the correlation between PGR and mir-4728 but failed to conclude the inner mechanisms, which deserved attention.

In summary, we showed a novel role of mir-4728 in the diagnosis and prognosis prediction of breast cancer. Mir-4728 represents an excellent biomarker for the prediction of the HER2 expression status in breast cancer patients. Meanwhile, mir-4728 can predict the poor prognosis of high tumor burden breast cancer patients and may evaluate the therapeutic effect of targeted therapy for breast cancer patients. ESR, but not PGR, is confirmed as the direct target of miR-4728-3p. This work shed light on the clinical use of mir-4728, which can become a valuable biomarker for the diagnosis of HER2 status and the assessment of therapeutic effects on breast cancer if given a multi-center prospective clinical study validation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

XZ and TR designed the theme and organized the research. TR, XL, and AX conducted the data collection and analysis. AX, JG, NT, XJ, and JL contributed to the data analysis. TR and XZ performed the experiments. XZ and TR prepared the original draft. All the authors have read and revised the final manuscript.

FUNDING

The results shown are based on the data obtained from the TCGA database. This study is supported by The Construction Fund of Medical Key Disciplines of Hang Zhou, China.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2022.818493/full#supplementary-material

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frontiers | Frontiers in Molecular Biosciences

REVIEW
published: 06 July 2022
doi: 10.3389/fmolb.2022.834149

The Multi-Functional Roles of CCR7 in Human Immunology and as a Promising Therapeutic Target for Cancer Therapeutics

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An important hallmark of the human immune system is to provide adaptive immunity against pathogens but tolerance toward self-antigens. The CC-chemokine receptor 7 (CCR7) provides a significant contribution in guiding cells to and within lymphoid organs and is important for acquiring immunity and tolerance. The CCR7 holds great importance in establishing thymic architecture and function and naïve and regulatory T-cell homing in the lymph nodes. Similarly, the receptor is a key regulator in cancer cell migration and the movement of dendritic cells. This makes the CCR7 an important receptor as a drug and prognostic marker. In this review, we discussed several biological roles of the CCR7 and its importance as a drug and prognostic marker.

Keywords: CC-chemokine receptor 7, CCL19, CCL21, immune tolerance, drug target, prognostic marker

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Molecular Diagnostics and Therapeutics, a section of the journal Frontiers in Molecular Biosciences

Received: *13 December 2021*
Accepted: *26 May 2022*
Published: *06 July 2022*

Citation:
Alrumaihi F (2022) The Multi-Functional Roles of CCR7 in Human Immunology and as a Promising Therapeutic Target for Cancer Therapeutics. Front. Mol. Biosci. 9:834149. doi: 10.3389/fmolb.2022.834149

BACKGROUND

To ensure efficient functioning of the immune system, the interaction between immune and non-immune cells is imperative (Stockis et al., 2019). These cellular encounters greatly rely on the cells' ability to migrate to a defined site (Förster et al., 2008). The trafficking of immune cells is regulated by key regulators known as chemokines (Hughes and Nibbs, 2018). Some of these chemokines are produced during infection, while others such as CC-chemokine ligand 21 (CCL21) and CCL19 are expressed every time and function to control cell movement (Förster et al., 2008). Both CCL21 and CCL19 act as sole ligands for a CC-chemokine receptor 7 (CCR7) (Hauser and Legler, 2016). The CCR7 protein is the product of the CCR7 gene and is recently designated as a cluster of differentiation 197 (CD197) (Cuesta-Mateos et al., 2021). Different cells of the immunity system are responsible for CCR7 expression and along with its ligands play a key part in localizing antigen-presenting dendritic cells and T cell subpopulation to lymph nodes, where the cells establish close contacts to drive activation of antigen presentation (Lipscomb and Masten, 2002). The CCR7 is implicated in optimal induction of protective immunity and also for the stimulation of peripheral tolerance induction and immunity response regulation by $CD4^{+}CD25^{+}$ regulatory T cells (Cools et al., 2007) (Kondo et al., 2019).

CCR7 AND ITS BINDERS

There are two ligands for CCR7; CCL19 and CCL21. In order to have avid binding to glycosaminoglycans (Rot, 2010), CCL21 has a unique 12 basic amino acid patch in the long C-terminal tail of 32 residues (Proudfoot et al., 2017). The binding event is a prerequisite for effective

presentation of CCL21 on endothelial cell surfaces (Miyasaka and Tanaka, 2004). The CCL21 presentation is specifically carried out by podoplanin, which is a proteoglycan expressed by different cell types and might regulate CCL21 availability (Johnson and Jackson, 2010). In mouse experimentation, due to gene duplication, two functional CCL21 variants have been noticed (Zlotnik et al., 2006). One is CCL21-Leu with leucine at position 65 and is expressed by the colon, lung, stomach, skin, and heart (Schumann, 2011). On the other hand, CCL21-Ser is expressed by lymph nodes, thymus, and spleen (Mori et al., 2001). It is interesting to know that the human genome only encodes CCL21-leu and not CCL21-Ser (Hauser and Legler, 2016). The CCL21 in humans and mice is yielded by fibroblastic reticular cells and endothelial venules (Link et al., 2011) (Al-Jokhadar et al., 2017) (Seth et al., 2011). The CCR7 is made of seven transmembrane domain containing proteins and facilitates its signaling pathways through heterotrimeric G proteins (Maghazachi, 2005). The expression of CCR7 is carried out by thymocytes, mature and semi-mature dendritic cells, regulatory T-cells, naïve T-and B-cells, and central memory T-cells (Schneider et al., 2007). In addition, CCR7 expression is carried out by different malignant cells. For CCR7, CCL19 and CCl21 had shown the same binding affinities though they initiate various singling pathways leading to different impacts (Müller et al., 2003). The CCL19 in contrast to CCL21 activates CCR7 internalization and phosphorylation, which shorten the time span of CCR7-mediated cell responses to CCL19 (Hauser and Legler, 2016). Similarly, the CCL19 can desensitize the CCR7 in its subsequent response to CCL21 ligation (Zidar et al., 2009). Together with CCL25, CCL19 and CCL21 have to potential to bind with high affinity to CC-X-chemokine receptors, which act as chemokine interceptors by internalizing ligands and transporting them (Förster et al., 2008).

MULTIFUNCTIONAL ROLES OF CCR7 IN HOST IMMUNOLOGY

Significance of CCR7 in Immune Cell Regulation

The localization of immune cells to defined functional compartments is controlled by CCR7-mediated signals (Worbs and Förster, 2007). The majority of the T-cells such as memory, naïve, and regulatory T-cells are allowed to penetrate lymph nodes involving a stepwise procedure of interaction of adhesion to endothelial cells (Nolz et al., 2011). In mice experimentation, CCR7 deficiency results in lack of T-cells in lymph nodes (Okada et al., 2002). It was also observed that T-cells are unable to home the lymph nodes but localize to the spleen in the absence of functional CCR7 (Sharma et al., 2015). The B cells in the CCR7-deficient case have the potential to migrate to splenic white pulp and lymph nodes (Katagiri et al., 2004). Though the CCR7 as a receptor of lymph node homing is well-established, evidence suggesting its role in lymphocyte recirculation is also very real (Link et al., 2007). The emigration of T-cells to peripheral tissues and entrance of T-cells to lymph nodes is also a CCR7-dependent step (Ebert et al., 2005). The dendritic cells are present as sentinels in the skin and alimentary, respiratory, and urogenital tracts (Hendry et al., 2017). The activation of dendritic cells by an infectious agent or inflammatory events drives the cells to undergo maturation, resulting in major changes in antigen uptake and presentation (Stockwin et al., 2000). The maturation of dendritic cells can be categorized by the higher expression of CCR7 and CD80, CH83, and CD86 (Chiesa et al., 2003). Very less is known about the exact mechanism of how trafficking of dendritic cells *via* different lymphatic events occurs (Alvarez et al., 2008). Furthermore, it is still under investigation how CCR7 and its ligands mobilize the dendritic cells (McKenna et al., 2005). Both the wild and CCR7-deficient mice were reported to have the same dendritic cell numbers in the peripheral organs (del Rio et al., 2007). This implies that CCR7 has no direct involvement in dendritic cell progenitor recruitment to mucosal and skin surfaces (Cutler and Jotwani, 2004). The migration ability of differentiated dendritic cells from bone marrow to lymph nodes is a major hinderance in CCR7-deicient mice (León et al., 2005). It is also analyzed that the turnover of dendritic cells from the lung, skin, and intestine depends on the CCR7 (Hintzen et al., 2006). In *in vivo* studies, it has been demonstrated that CCL19 and CCL21-Ser derived from lymph nodes take part in activating dendritic cell relocation into the lymph nodes (Denton et al., 2014). CCL19 and CCL21 are needed for dendritic cell guiding in the lymph nodes. Furthermore, research findings speculated that CCL19 and CCL21 are capable of priming T cells along with driving the dendritic cell migration. The uptake of antigens by mature dendritic cells is facilitated by CCR7 ligands (Seubert et al., 2008). A graphical illustration of the stepwise process of lymphocyte homing to the lymph nodes is provided in **Figure 1**.

The Role of CCR7 in Lymph-Node Homing

Upon entrance into the lymph node, naïve T-cells begin to migrate in a random walk pattern in the paracortical T-cell-rich area (Krummel et al., 2016) (Weninger et al., 2003). The CCR7-deficient T cells in popliteal lymph nodes have shown 30% reduced velocity as well as 50% reduced motility coefficient (Worbs et al., 2007). Furthermore, a notable dichotomy has been observed within the lymph nodes for chemokine receptor usage (Garcia et al., 2005). The CCR7 activates signals that allow the cell to migrate into the T-cell areas (Arnold et al., 2007). Upon activation, the follicular B-cells upregulate CCR7 and downregulate CXXR5. The differential chemokine receptor expression drives the movement of follicular B-cells to the T-cell zone to get help from $CD4^+$ T cells (Eisenbarth et al., 2021). The expression of CCR7 on $CD4^+CXCR5^+$ follicular T cells permits the cells to enter into B cell follicles for providing help in antibody production and class switching (Hardtke et al., 2005). Overall, it can be concluded that CCR7 is a lymph-node receptor for dendritic cells and T-cells.

The Role of CCR7 in Immune Tolerance

The weak immunity in CCR7-deficient mice after administration of a model antigen further illustrates the multifaceted role of CCR7 and its ligand molecules on the immune system and their vital importance in paracortical area organization in the lymph

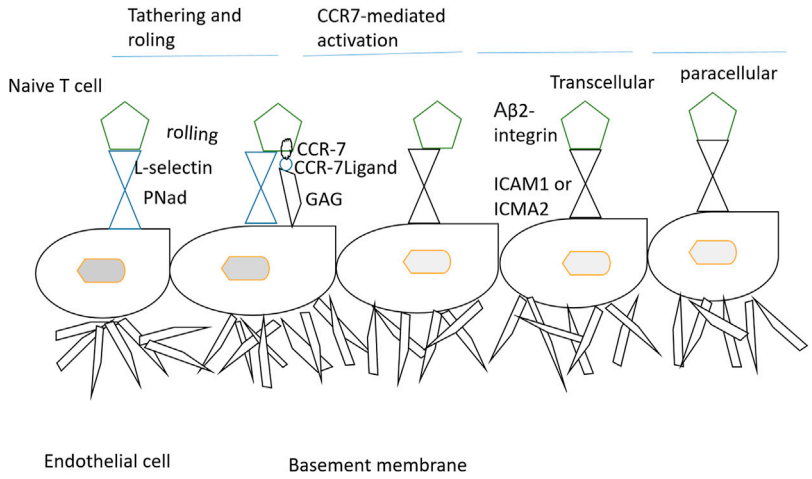

FIGURE 1 | Stepwise mechanism of lymphocyte homing to the lymph nodes. The T-cells when emerging from the blood enter the peripheral lymph nodes through the tethering and rolling mechanism, activation of CCR7, firm arrest, and transendothelial migration. At first, L-selection of lymphocyte binds with peripheral node addressins (PNAd) and sialomucins on high endothelial venules (HEVs). The interaction results in T-cell attachment to HEVs and results in cell rolling. The rolling cells then interact with CCL21/CCL19 and thus are immobilized by glycosaminoglycans (GAGs). The signals from CCR7 and blood flow force induce conformational changes in $\alpha_L\beta_2$-integrins, thus allowing firm binding to intracellular addressin cell adhesion molecular 1 (ICAM1) and ICAM2. CCR7 also activates $\alpha_L\beta_2$-integrin mucosal addressin cell-adhesion molecule 1 (MAdCAM) (Förster et al., 2008).

node (Worbs and Förster, 2007). Studies have also shown that the CCR7-deficient mice impaired humoral immune responses in case of low antigen against replicating virus and high amount of virus glycoproteins (Scandella et al., 2007). These findings imply that when the antigen is sparse, CCR7 holds significant importance in interactions among immune cells (Qi et al., 2006). In some cases, the CCR7-mediated interactions are bypassed in providing adaptive immunity against a pathogen (Moretta et al., 2008). This was highlighted in CCR7-deficient mice where neutralizing immune responses were seen mounted against the choriomeningitis virus (Junt et al., 2008). It was also observed that for priming the naïve MHC-class-Ia-restricted $CD8^+$ T cells, the presence of CCR7 is required, whereas MHC-class-II-restricted $CD4^+$ T cells and naive MHC-class-Ib-restricted $CD8^+$ T cells do not require chemokine receptor (Tzelepis et al., 2007). In addition, it was revealed that repeated administration of tetanus toxoid stimulated humoral and full-blown cellular immunity in CCR7-deficient mice (Macpherson et al., 2008). In auto-immune encephalitis, allergic asthma, and inflammatory bowel disease, substantial immune responses in mice were developed in the absence of CCR7 and its ligands (Griffith et al., 2014). The nonstop migration of dendritic cells from the periphery is a critical step in inducing immune tolerance in response to any food or environmental antigen (Zhang et al., 2021). The migration of tolerogenic or semi-mature dendritic cells into draining lymph nodes depends on CCR7 expression (Förster et al., 2012). This was tested in CCR7-deficient mice whether dendritic cell–mediated transportation of harmless antigens is required for peripheral tolerance (Worbs et al., 2006). The use of intravenous or subcutaneous injection of model antigen ovalbumin in wild-type mice results in systematic non-responsiveness toward model antigen ovalbumin (Steenblock et al., 2009). The mesenteric lymph node was identified as a site of antigen presentation to T cells (Buettner and Bode, 2012) (Jang et al., 2006). Further clarity on the point was obtained from studies where antigen delivery to the respiratory tract is carried out by intratracheal instillation or inhalation (Lombry et al., 2004). The antigen was labeled with fluorochrome to monitor its *in vivo* and *ex vivo* experimentations. The CCR7-deficient mice showed no effect of model antigen ovalbumin aerosol on reporter T-cells (Förster et al., 2008). Therefore, it can be summarized that under homeostatic conditions, the dendritic cells at mucosal sites can induce tolerance in the presence of CCR7 by sampling antigens and transporting them to draining lymph nodes to be efficiently presented to T-cells (Seth et al., 2011).

Suppression of the host immunity through forkhead box P3 (FOXP3) T-cells is considered an alternative method for efficient peripheral immune tolerance to foreign and self-antigens (Nishikawa and Sakaguchi, 2010). The regulatory T-cells can be naturally produced in the thymus when CCR7 is absent. In both wild and CCR7-deficient mice, the total number of $FOXP3^+$ T-cells is the same (Schneider et al., 2007) (Smigiel et al., 2014). This can be rational that *in vivo* the cells are unable to reach the lymph nodes and incapable of placing themselves in the T-cell zone (Groom et al., 2012). In the lymph nodes, the exact mechanism behind the regulatory T-cell suppressive activity is still unknown (Wei et al., 2018). The regulatory T-cell homing T-cell zone of the lymph node is mediated by CCR7, proliferates, and expands when they encounter their cognate antigen (Schneider et al., 2007). Reduced number of activated T helper cells due to CCR7-dependent presence of regulatory T-cells is observed (Bayry et al., 2007). Schematically, the CCR7-mediated immune tolerance is presented in **Figure 2**.

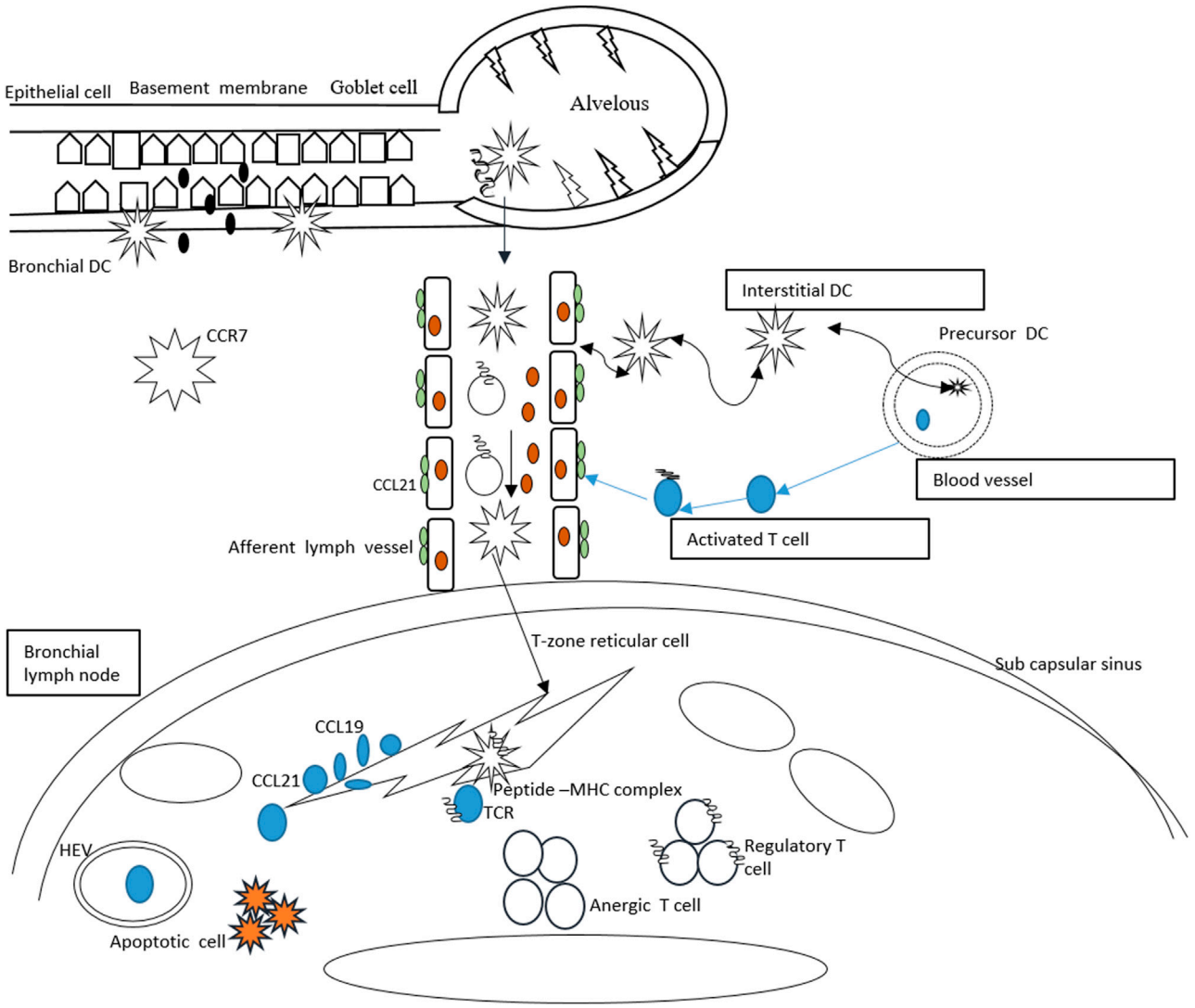

FIGURE 2 | CCR7-mediated tolerance in response to inhaled antigens. The dendritic cells enter the lungs and produce interstitial and bronchial dendritic cells. The bronchial dendritic cells carry the antigens and upregulate the CCR7 and move toward lymphatic vessels to activate CCL21. The dendritic cells are passively transported into the draining lymph node. The dendritic cells then present antigens to the naïve T-cells and *via* endothelial venules, the T-cells penetrate into the lymph node. Migration of T-cells on reticular cells results in expression of CCL19/21.

The Role of CCR7 in Autoimmunity and Lymphoid Neogenesis

It has been observed that the absence of CCR7 is directly associated with the onset of spontaneous autoimmunity. This was evaluated in CCR7-deficient mice where lymphocyte infiltration was reported in different peripheral organs along with high auto-antibody titer resulting in IgG deposition in renal glomeruli. Further investigation reported that the emergence of autoimmunity is the product of ineffective negative selection of autoreactive T cells, defective regulatory T cell function, and lack of proper peripheral tolerance maintenance. It was also noticed that CCR7-deficient mice develop lymphoid at sites such as the stomach, lung, and colon; however, it is not exactly known about the extent of ectopic lymphoid structure contribution to autoimmunity establishment and maintenance. In the absence of CCR7, spontaneous lymphoid neogenesis is also witnessed emphasizing the fact that CCR7 is not needed for the process. Tertiary lymphoid structures are also formed due to transgenic expression of CCR7 in the pancreas and thyroid. Furthermore, tertiary lymphoid structure development in different organs is correlated with CCL21 ectopic expression in infection and autoimmunity. This process is hypothesized to be mediated by CCR7 as tertiary lymphoid structures are not formed in CCR7-deficient mice expressing CCL21. The functioning of CCR7 in regulatory T-cells is presented in **Figure 3**.

The Role of CCR7 in Thymus

The thymus is an important organ that maintains the pool of peripheral T cells. The CCR7 is revealed to be vital for organizing

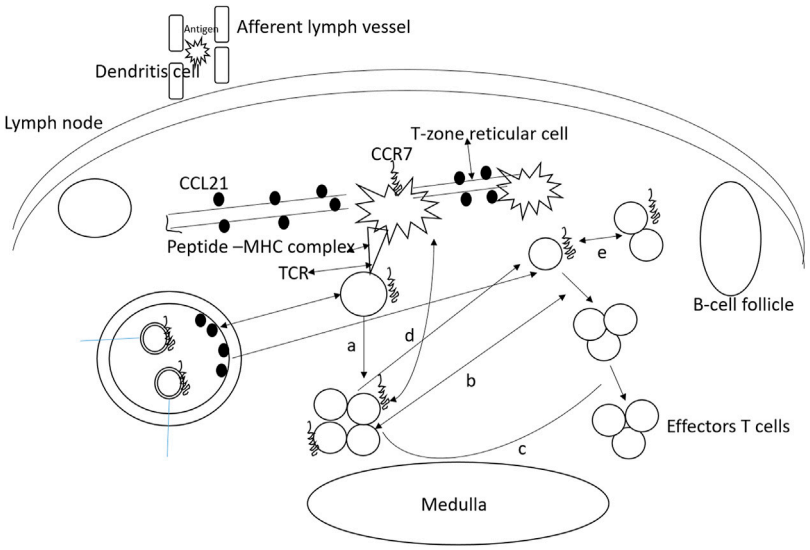

FIGURE 3 | Functioning of CCR7 in regulatory T-cells. Almost all regulatory T-cells express CCR7 and use it for entering into lymph nodes. These cells homing to the lymph node allow their interaction with the antigens on the dendritic cells. As a result, the regulatory T-cells proliferate and expand upon presentation with the antigen. **(A)** Interfere with naïve T helper cell proliferation, **(B)** low number effector cells and inhibits differentiation, **(C)** regulatory T-cells exert suppressive activities by targeting dendritic cells, and **(D)** T-cells. **(E)** naive T cell conversion to regulatory T cells in the presence of low amount of antigens.

migratory events of cells in the thymus (Bunting et al., 2011). During embryogenesis, the CCL21 is reported to be involved in fetal hematopoietic progenitor recruitment in developing organs (Liu et al., 2005). The statement can be supported by the fact that CCR7-deficient mice are found to have a reduced number of thymocytes (Laan et al., 2009). Studies have also revealed that mouse overexpression of CCX-CKR possesses a low number of hematopoietic precursors in the thymic region (Bunting et al., 2013). The CCL19 and CCL21 in the adult thymus are not restricted to any compartment and are detectable in the medulla and cortex (Kwan and Killeen, 2004). As a result, CCR7 ligands are capable of guiding developed thymocyte migration through thymic compartments (Kwan and Killeen, 2004). The CD4 and CD8 expression in early progenitors is absent, and the cells are referred to as double negative cells (Ceredig and Rolink, 2002). The expression of CCR7 is prominent in the double-negative subpopulation cells ($CD44^{hi}$ $CD25^{int}$) (Bulati et al., 2014). About fifty percent of these cells express CCR7 reflecting the role of CCR7 in cell migration from cortico–medullary junction (Braun et al., 2011). Recently, the CCR7 role in the translocation of double-positive thymocytes has been studied (Kwan and Killeen, 2004). The CCR7 expression is abundant in single-positive populations (Castro et al., 2014). These cells are found in high concentrations in the medulla. Interestingly, the immature $CD4^{+}$ single-positive cells express very low CCR7 (Kurobe et al., 2006). On the other hand, immune cells that do not undergo negative selection and are mature produce a high amount of CCR7 (McDonald et al., 2015). Another important role of CCR7 expression is the mature thymocyte positioning near blood vessels prior to leaving the thymus (Kwan and Killeen, 2004). The thymus morphology disruption is the result of central tolerance breakdown and autoimmunity development (Lomada et al., 2007). During T cell production in the thymus, the absence of CCR7 signaling contributes to autoimmunity manifestation in CCR7-deficient mice. Along with this, it is also elucidated that CCR7-deficient mice reported defects in negative selection, which might be due to impaired T cell receptor stimulation, and this further signifies the contribution of CCR7 in central tolerance maintenance (Davalos-Misslitz et al., 2007). The role of CCR7 in the migration of thymocyte is given in **Figure 4**.

ROLE OF CCR7 IN TUMOR GROWTH AND EXPANSION

CCL19 and CCL21 are mostly expressed during the growth of lymphatic vessels and also in other lymphatic organs (Krishnamurty and Turley, 2020) (Wirsing et al., 2018). Disparate CCL19 and CCL21 bind to glycosaminoglycans (GAGs) and immobilize on endothelial cells (Jørgensen et al., 2021). Remarkably, literature reported that CCR7 stimulation with both CCL21 and CCL19 ligands enhances G-protein activation, migration of cells, signaling pathway of the ERK 1/2, and mobilization of calcium (Rizeq and Malki, 2020). Desensitization of the CCR7 and its activation of ERK are mainly facilitated by β-arrestin, suggesting that the effects of CCL19 may be more transitory than with CCL21 cytokines (van Gastel et al., 2018).

Moreover, semi-mature, CXCR4/CXCL12 expression is directly associated with the directing of cancer cells to the lungs, liver, and lymphatic nodes (Liu et al., 2020). The high-level expression of the CCR7/CCL21 axis has mostly related to metastasis lymph nodes regions, while it also plays a vital role in the progression of several different types of other malignancies, such as breast (Cabioglu et al.,

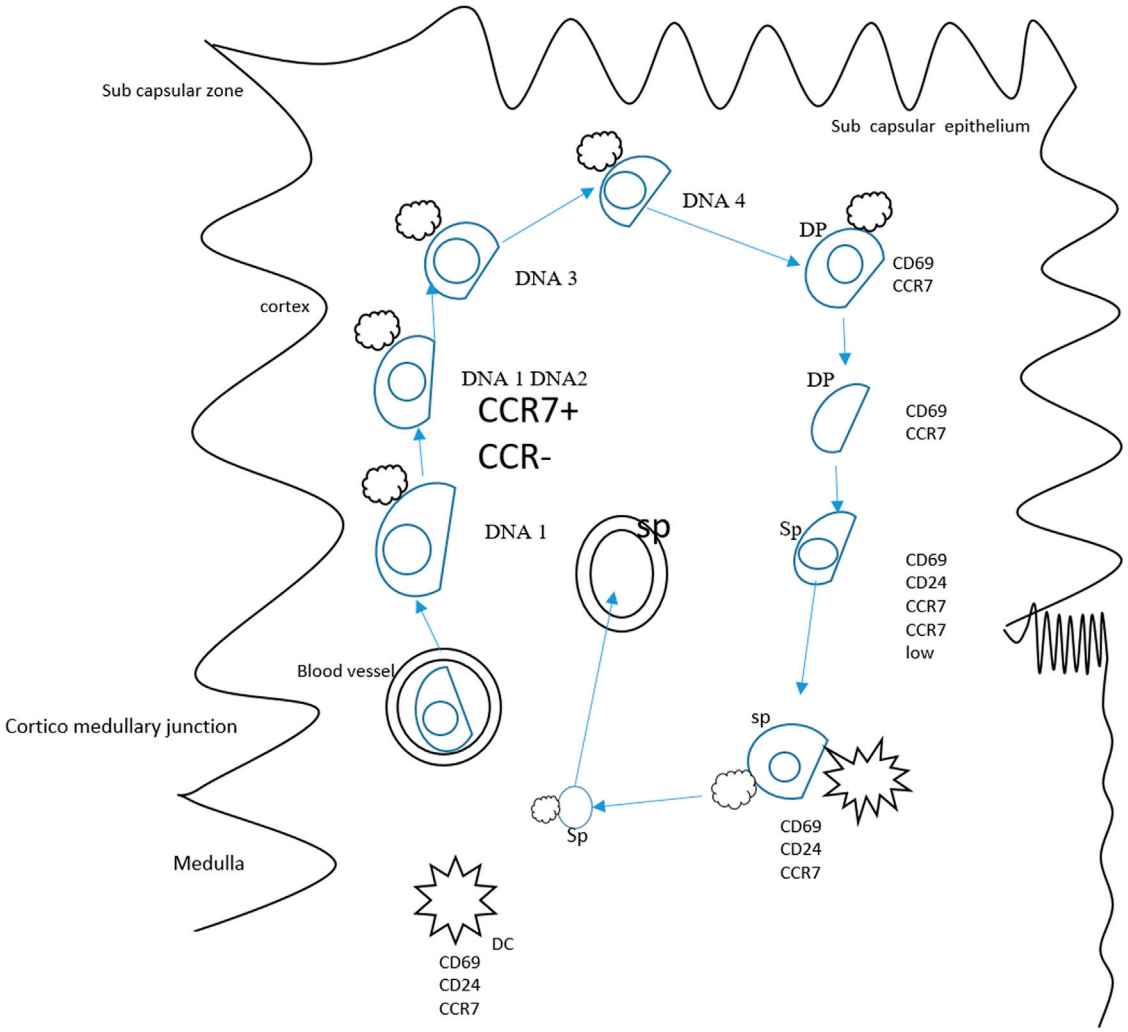

FIGURE 4 | Role of CCR7 in the migration of thymocytes. Thymocyte progenitors derived from bone marrow travel to the thymus. As the CD4/8 lacks expression, the cells are known as double-negative cells. The DN1 cells differentiate at the thymic entry site, and transformation to DN2 occurs in the mid cortex. The DN3 thymocyte differentiation happens while cells migrate to the outer cortex and developed into DN4 cells in the sub-capsular zone. The double-negative dendritic cell transition to the double-positive phase is accomplished in reverse migration and the double-positive thymocytes enter the medulla. In the medulla, positively double-positive cells mature and result in the production of $CD4^+/CD8^+$. A small population of double-positive cells expresses CCR7 and might drive the migration of double-positive cells to the medulla from the cortex. The CCR7 also plays a key role in mature single positive CD62L cells and guides the maturation of these cells. In this period, thymocytes interact with dendritic cells and medullary and thymic epithelial cells, deleting auto-reactive thymocytes and guiding positive selection.

2005), gastro (Mashino et al., 2002), melanoma (Takeuchi et al., 2004), neck (Tsuzuki et al., 2006), lung (Takanami, 2003), hepatocyte (Yang et al., 2018), cervical (Wang et al., 2021), thyroid (Wagner et al., 2008), tonsillar (Takeuchi et al., 2004), colon (Li et al., 2011), and prostate cancers (Berndt et al., 2013) as tabulated in **Table 1**. In many reported cases of these types of malignant cancers, increased size of tumor and invasions were due to CCR7 (Kodama et al., 2007).

THE ROLE OF CCR7 IN CANCER CELL MIGRATION

Cellular migration *in situ* and *ex situ* is dependent on the biochemical and physical properties of cells. For cells to come out from the blood veins and adhere to the endothelial layer, the chemokines must need to bind with GAGs located in the extracellular matrix (ECM) (Eble and Niland, 2009). There is an electrostatic type of interaction somewhere in the C-terminal region of the chemokine and is positively charged because of lysine and arginine, whereas GAGs possess a negative charge because of the presence of sulfate and carboxylate residues (Severin et al., 2010). Recent research works have reported that body cells can sense the physical and environmental stimuli and respond by altering cellular expression (Kraning-Rush et al., 2012). In addition, chemokines can enhance relocation toward an increasing meditation of a chemo-attractant (Yang et al., 2005). In mature dendritic cells, for example, immobilized CCL21 causes outgrowth of cell and integrin activation, while mobilized CCL19 and CCL21 increase the chemotaxis process (Haessler et al., 2011).

TABLE 1 | Involvement of CCR7 in different human cancers.

Type	Role	References
Bladder cancer	Invasion, migration, proliferation, and poor prognosis	(Mo et al., 2015; Xiong et al., 2017)
Breast cancer	Lymphogenesis, metastasis, and actin polymerization	(Li et al., 2017; Xu et al., 2017; Rizeq and Malki, 2020)
Colorectal cancer	Metastasis and poor prognosis	(Gao et al., 2019; Nagasawa et al., 2021)
Cervical cancer	Metastasis and poor prognosis	(Dai et al., 2017; Tian et al., 2021)
Gastric cancer	Metastasis and poor survival	(Du et al., 2017; Mao et al., 2017)
Lymphomas	Tumor dissemination and poor prognosis	(Li et al., 2018; Du et al., 2019)
Lung cancer	Metastasis and tumor dissemination	(Kwiecień et al., 2019; Salem et al., 2021)
Head and neck cell carcinoma	Metastasis	(Al-Jokhadar et al., 2017; Liu et al., 2018)
Prostate cancer	Metastasis, tumor growth, and lymphatic metastasis	(Makino et al., 2019; Rizeq and Malki, 2020)
Esophageal cancer	Poor prognosis, angiogenesis, and metastasis	(Irino et al., 2014; Yang et al., 2018)
Melanomas	Metastasis and poor outcome	(Takeuchi et al., 2004; Legler et al., 2014)

The migration of WBC and CCR7 (+) malignant cells spreading into secondary lymphatic organs is specifically regulated through the interaction of chemokine–chemokine receptors in the environment, and T-cell migration–mediated CCR7-proteins within SLT is very crucial for activation of T-cells in order to generate adaptive immunity (Castriconi et al., 2018). Exploring the migration response of CCR7 proteins coding T-cells within certain types of chemokine environment will facilitate a better understanding of the process of T-cell migration (van der Woude et al., 2017). A function examination of CCR7 in chemotaxis cells may also be helpful in understanding its function in cancer spreading (Wu et al., 2009).

CCR7 is of particular attention in understanding metastasis due to CD4 positive T-cells and dendritic cells needing expression of CCR7 to migrate with the lymphatic tissue (Roberts et al., 2016). The function of lymphatic organs as the extracellular fluids flow sink; it has been assumed that interstitial fluids flow and CCL21 role in conjunction to monitor the migrating of the cancer cells to lymphatic vessels in the development of metastases of cancer (Angeli and Randolph, 2006).

Several studies have revealed that CCL19 and CCL21 can vigorously drive the chemotaxis migration of CCR7-expressing tumor cells (Kabelitz and Wesch, 2003). Furthermore, CCL21 has also been observed to provoke the production of new lymphoid-like structures (Pitzalis et al., 2014). But, the function of CCL21 throughout tumor progression time remains slightly debatable. CCL21 is one of the effective chemo-attractant for tumor-penetrating white blood cells (Rizeq and Malki, 2020). The latest clinical research study described an increased outcome related to increased infiltration of CCR7 (+) T lymphocytes in advanced colon cell carcinoma (Banerje e et al., 2021). In stomach cancer expression of CCR7, early tumor cells were investigated as the most significant component in the determination of lymph node metastasis in cancers (Nagasawa et al., 2021).

CCR7 AND ANGIOGENESIS

CCR7 has also been linked to the formation of a new lymphoid vessel in breast carcinoma patient samples, but the actual mechanism is still unknown (Leong et al., 2021). This lymphoid angiogenesis is mainly facilitated by VEGF-C and the receptor of VEGFR-3 (Angeli and Randolph, 2006). Certainly, the high-level expression of this growth factor is well-reported in increased lymphoid node metastasis type of cancer (Ray and Cleary, 2017). Remarkably, there are several other types of reported studies signifying that each time cancer cells express CCL21 and increase the level of white blood cell recruitment in a specific subpopulation of T–cells CD8 positive and dendritic cells (Rizeq and Malki, 2020).

CCR7 AS A POTENTIAL DRUG TARGET

The transmembrane protein CCR7 is correlated with the spread of cancer to the lymph nodes in colon cancer and thus considered a beneficial therapeutic target (Salem et al., 2021). The structure of CCR7 attached to allosteric antagonist Cmp2105 was explained by Jaeger, Bruenle, and their colleagues (Jaeger et al., 2019). The CCR7 was fused with the 52.8 kDa; protein sialidase NanA to ensure its crystallization, and the crystals were distributed to a resolution of 2.1 Å. Cmp2105 was added to the CCR7 which made it more stabilized, and the IC_{50} of Cmp2105 in membrane-based competition assays was measured by radiolabeled CCL19, and its measured value was 35 NM. Surprisingly, the structure showed that Cmp2105 was found inside an intracellular space at the ends of transmembrane (TM) helices. As compared to CX3CL1 and CCR2, Cmp2105 stabilizes an inactive confirmation of CCR7. The similarity search of the 3-D model of 2.3 million compounds using Cmp2105 resulted in the finding that there were 293 compounds with similar pharmacophores to the Cmp2105. The thermal stability assays identified the top two best matches. One of these two was navarixin, which is also called SCH-527123, and MK-7123 antagonist, which shows larger efficacy and solubility. As navarixin has noticeable antimetastatic activity in colon cancer, soCZzmer, and some other cancers, therefore it is now in phase II clinical trials. Because of this observed antagonistic activity, there is the possibility of navarixin being utilized for preventing metastasis, which may likely contribute to the CCR7 antagonism mechanism. Furthermore, this study of CCR7 attached to an antagonist may provide a good platform for additional investigation of some available CCR7 antagonists.

CCR7 AS A PROGNOSTIC MARKER

Different research reported CCR7 as a cancer marker, but its effects on the OS of cancer patients are still unknown because different studies have shown distinguished results even in the same type of tumor in different patients, for example, rectal cancer and lung cancer. It is also reported that CCR7 has no notable effects on OS in other tumor types such as gastric cancer and breast cancer and SCCHN (Salem et al., 2021). This study found that the association between CCR7 and the diagnosis of several tumors has not been explained and reviewed yet. So, they conducted a meta-analysis to issue valid medical resources on the diagnostic value of CCR7. This meta-analysis included 30 studies in which there were 3,413 patients having 15 different types of tumors. The conducted meta-analysis showed that higher expression of CCR7 can independently be used as an indicator of poorer OS in patients having a tumor. Increased level of CCR7 was also correlated with the worst PFS; but there was no evidence to detect the association of CCR7 with DFS, RFS, and DSS. To investigate the prognostic value of CCR7 in other tumors, further investigation of the subgroup for overall survival (OS) values was performed and because of limited available data, the subgroup analysis for other values was not performed. The results showed that upregulation of CCR7 magnificently lowered the OS of esophageal and gastric tumors patients. Furthermore, the overexpression of CCR7 indicated poor OS in patients having breast cancer, but this prediction was not significant. Over CCR7 expression in patients with lung cancer predicted an association with the best diagnosis. The numbers of samples were not sufficient, which makes the results insignificant and that was of course one of the limitations of the study. Another factor was the negative prognostic factor in patients having tumors in the urogenital system and digestive system. Due to the limited sample size, the association between expressing CCR7 and tumor prognosis is considered not convincing, which can be improved by enlarging the sample size and some further analysis of the association of CCR7 with the clinical prognostic values.

Apart from CCR7 as a prognostic marker in cancer, there were some shortcomings of the work. First, the number of samples was not sufficient; second, CCR7 expression cutoff values were not the same in all studies, which can decrease the efficacy of the results; and third, the HR values were obtained from survival curves which can produce a statistical error. Significant heterogeneity was shown in this meta-analysis and that could be considered in different important factors, for example, type of tumor, method of analysis, the source of the sample, and cutoff value.

The results of this meta-analysis suggested that in some types of tumors, the overexpression of CCR7 is correlated to the worst prognosis of tumor patients (Zu et al., 2019). Though the predictions show that in lung cancer and colon cancer, the CCR7 expression is related to prognosis, but these results need to be improved (Günther et al., 2005). It is concluded that CCR7 is a good indicator in tumors, and these results should be considered carefully.

CONCLUSION

The CCR7 and its ligands have received great attention in recent times due to their versatile functioning in regulating leukocyte function during immunological responses. The chemokine ability to convey signals that are remarkably versatile and specific makes them powerful modulators of immunological responses against diverse antigens. Considering the importance of CCR7, in this review, we seek to address the importance of CCR7 in immune cell regulation, lymph node homing, immune tolerance, different types of cancer, and CCR7 as a therapeutic and prognostic marker. The literature reported herein might attract the readers for expanding their knowledge of chemokines and a better approach to novel therapeutics in the near future.

AUTHOR CONTRIBUTIONS

FA planned this study, gathered the data, and prepared the whole review.

ACKNOWLEDGMENTS

The researcher would like to thank the Deanship of Scientific Research, Qassim University for funding the publication of this project.

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