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Published in:
Biocell

DOI:
10.32604/biocell.2022.016916

Print publication: 01/01/2022

Document Version
Publisher's PDF, also known as Version of record

Link to publication

Citation for pulished version (APA):
Thakur, P., Saini, R. V., Chhillar, A. K., Saini, N. K., Thakur, V. K., Siwal, S. S., & Saini, A. K. (2022). Alteration in the expression of microRNA-21 regulated target genes: Role in breast cancer. *Biocell*, *46*(2), 309-324. https://doi.org/10.32604/biocell.2022.016916

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Download date: 05. Nov. 2022

BIOCELL
2022 46(2): 309-324

Tech Science Press

Alteration in the expression of microRNA-21 regulated target genes: Role in breast cancer

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Key words: Non-coding RNA, microRNAs, Breast cancer, Tumor suppression, Biomarker

Abstract: Breast cancer, also recognized as the principal cause of cancer-related deaths among women, is the second most familiar and prevalent form of cancer. New diagnostic and prognostic biomarkers that are highly specific are urgently needed for its early prognosis. MicroRNAs (miRNAs), a class of non-coding RNAs, are known to control the biological processes involving transcription, post-transcriptional and covalent modifications, splicing, translation, cell differentiation, proliferation, apoptosis, cancer progression, and invasion. Any dysregulation in miRNA expression, demonstrating their oncogenic and tumor-suppressive functions, contributes to cancer progression. MicroRNA-21 (miR-21), an 'onco-miR' in breast cancer, is involved in tumor progression and metastasis by suppressing the activity of the target gene via its interaction with the 3'UTR of the target gene. The upregulation of miR-21 is observed in many instances of breast cancer. Our review aims to summarize the current understanding of miR-21 in the regulation of important cellular functions via regulation of its target genes. We discuss its biosynthesis, oncogenic function in breast cancer, and different methods used for its detection. This will increase the current understanding of the role of miR-21 in breast cancer tumorigenesis, which will offer a perception of using miR-21 as an early detection molecular prognostic and diagnostic biomarker and as a therapeutic target in breast cancer care.

Abbreviations

Her2: Human epidermal growth factor receptor 2
ncRNAs: Noncoding RNAs
miRNAs: MicroRNAs
piRNA: PIWI-interacting RNAs
circRNAs: Circular RNAs
lncRNAs: Long noncoding RNAs
miR-21: MicroRNA-21
pre-miR-21: Precursor miR-21
pri-miRNA: Primary-miRNA
SNP: Single nucleotide polymorphism
m6A: N(6)-methyladenosine
HNRNPA2/B1: Heterogeneous nuclear ribonucleoprotein A2/B1
RISC: RNA-induced silencing complex
AGO2: Argonaute 2
miRISC: miRNA-induced silencing complex
Dcp 1: Decapping protein 1
Dcp 2: Decapping protein 2
XRN1: Exoribonuclease 1
qRT-PCR: Quantitative reverse transcription polymerase chain reaction
NGS: Next-generation sequencing
PDCD4: Programmed cell death 4
TPM1: Tropomyosin-1
PTEN: Phosphatase and tensin homolog
MASPIN: Mammary serine protease inhibitor

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Received: 09 April 2021; Accepted: 07 June 2021

Doi: 10.32604/biocell.2022.016916 www.techscience.com/journal/biocell

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Bcl-2: B-cell lymphoma 2
LZTFL1: Leucine zipper transcription factor-like 1
PRMT5: Protein arginine methyltransferase 5
OSCC: Oral squamous cell carcinoma
2-DIGE: Two-dimensional differentiation in-gel electrophoresis
anti-miR-21: Antisense miR-21
PIP: Phosphatidylinositol phosphate
FAP: Fibroblast activation protein
EMT: Epithelial-mesenchymal transition
MAPK: Mitogen-activated protein kinase

Introduction

The second most complex and heterogeneous cancer which brings mortality in 45–55 years older women across the world is breast cancer (Hemmatzadeh *et al*., 2016; Wang *et al*., 2017; Becker, 2015; Quan *et al*., 2020; Ataollahi *et al*., 2015). Aging is one of the most significant recognized risk factors associated with breast cancer. However, other risk factors such as deficiency of iodine, high estrogen level, obesity, intake of alcohol, menopause, family history, physically inactive and chest radiation exposure are also involved in breast cancer (Yager and Davidson, 2006; Steiner *et al*., 2008; Stoddard *et al*., 2008; Ataollahi *et al*., 2015). Breast cancer occurs in tissues that include mammary glands and ducts, resulting in lumps, swelling, redness, irritation in breast skin and nipples discharge (Ataollahi *et al*., 2015). The breast cancer cells are categorized into six major subtypes based on their phenotype and gene expression profile, including i) luminal A, ii) luminal B, iii) tumor enriched with human epidermal growth factor receptor 2 (also known as Her2), iv) basal-like (triple-negative), v) normal-like, and vi) claudin-low subtype (Singh and Mo, 2013; Sørlie *et al*., 2003; Eroles *et al*., 2012).

It is believed that the mortality rate due to breast cancer can be reduced through the early diagnosis and detection of these six subtypes (Imani *et al*., 2017). The mammographic screening tool is the most operative tool used for the early diagnosis of tissue-based tumors in the breast (Taplin *et al*., 2008; Imani *et al*., 2017). But this tool has certain limitations such as high false-positive results, low reactivity, and low preciseness and, not adequate to diagnose the subtypes, so there is a need for new diagnostic and prognostic biomarkers that are highly specific (Adhami *et al*., 2018; Imani *et al*., 2017). Various non-coding RNAs (ncRNAs) that involve microRNAs (miRNAs), PIWI-interacting RNAs (piRNA), circular RNAs (circRNAs), and long non-coding RNAs (lncRNAs) are found to regulate biological processes like transcription, post-transcriptional modifications, covalent modifications, splicing, and translation in the metastasis phase of breast cancer cells (Klinge, 2018). Of all the ncRNAs studied in breast cancer, miRNAs are the most thoroughly studied and have diagnostic potential (Lo *et al*., 2016).

miRNA in breast cancer

miRNAs perform a crucial function in cancer research as predictive, diagnostic and, prognostic biomarkers because of their regulatory roles in vital processes of life like cell differentiation, proliferation, apoptosis, cancer progression and invasion (Kim, 2005; O'Day and Lal, 2010; Yu and Cheah, 2017). miRNAs are 15-27 nucleotides, short endogenous ncRNA molecules that are well known for post-transcriptionally regulating gene expression in eukaryotes by base pairing with protein-coding mRNA genes 3'-UTR (Feng and Tsao, 2016; Han *et al*., 2016; Wilczynska and Bushell, 2015; Adhami *et al*., 2018). Around half of the human miRNAs are placed on human cancer-related chromosomal regions (fragile sites), enabling them to alter the tumor suppressor or oncogenic pathways (Calin *et al*., 2004; Hemmatzadeh *et al*., 2016; Tang *et al*., 2012; Casalini and Iorio, 2009; Croce, 2009). Various studies have shown the miRNAs involvement as oncogenic/tumor suppressor in the pathogenesis of breast cancer as defined in Tab. 1 (Zhu *et al*., 2008; Si *et al*., 2007; Yan *et al*., 2008; Kong *et al*., 2012; Hassan *et al*., 2012; Fabian and Sonenberg, 2012).

In breast cancer, microRNA-21 (miR-21) is the key miRNA used in the invasion process and facilitates tumor progression and metastasis (Han *et al*., 2012a; Han *et al*., 2012b). miR-21, also identified as MIR21, hsa-mir-21, miRNA21, MIRN21, is located on chromosome 17q21.3 encoding 72-nt long precursor miR-21 (pre-miR-21) (Abdel-hamid *et al*., 2015; Hemmatzadeh *et al*., 2016). By targeting multiple tumor/metastasis suppressor genes, miR-21 functions as an oncogene (Abdel-hamid *et al*., 2015; Hemmatzadeh *et al*., 2016; Selcuklu *et al*., 2009). The upregulated expression of miRNA-21 has been correlated with lymph node metastasis, advanced tumor levels and poor prognosis; therefore, it can act as a prognostic marker in breast cancer (Yan *et al*., 2008; Hemmatzadeh *et al*., 2016; Lee *et al*., 2011; Li *et al*., 2016; Shen *et al*., 2015).

Biosynthesis of miRNA

In the nucleus, the RNA polymerase II transcribes non-coding miRNA gene into 100-120 nt long hair pin structured primary-miRNA (pri-miRNA) (Petri and Klinge, 2020; Lee *et al*., 2004; Yu and Cheah, 2017) (as shown in Fig. 1). The enzymes DROSHA (a class 2 ribonuclease III) and DGCR8 (dsRNA-binding protein) cleave the hair pin structure of pri-miRNA to produce 70 nt pre-miRNA (Filippov *et al*., 2000; Gregory *et al*., 2004; Denli *et al*., 2004). *DROSHA* (encoded by miRNA machinery gene) is responsible for processing miRNA in the initial phase (Khan *et al*., 2014). The pri-miRNA is further modified by the N(6)-methyladenosine (m6A) post-transcriptionally, stimulating the beginning of the miRNA biosynthesis process (Alarcón *et al*., 2015a). The heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2/B1) attaches to m6A in pri-miRNA, assisting DROSHA's transformation of pri-miRNA into pre-miRNA (Alarcón *et al*., 2015b). After trimming of pri-miRNA, Exportin 5 (encoded by *XPO5*) or Exportin 1 (encoded by *XPO1*) exports pre-miRNA to the cytoplasm for further processing by the Dicer enzyme (Sheng *et al*., 2018). *DROSHA* plays a very significant role. The single nucleotide polymorphism (SNP) located in the 3'UTR of *DROSHA* rs10719 has been linked to breast cancer risk because it abrogates the hsa-miR-1298 binding site in *DROSHA*, thus affecting its expression (Khan *et al*., 2014).

TABLE 1

Oncogenic and tumor suppressor miRNAs target genes in breast cancer

miRNA	Target Genes	Role	References
Oncogenic miRNAs			
miR-21	*Bcl-2, PDCD4, TPM1, PTEN, MASPIN, LZTFL1*	Apoptosis; cell proliferation; β-catenin nuclear signaling; EMT process regulator	Si *et al.*, 2007; Zhu *et al.*, 2007; Frankel *et al.*, 2008; Qi *et al.*, 2009; Zhu *et al.*, 2008; Gong *et al.*, 2014; Wang *et al.*, 2019
miR-155	*RHOA, SOX1, SOCS1, MMP16, PTEN, DUSP14, PIK3R1, FOXO3a, ZEB2, TSPAN5*	TGF-β/Smad pathway; cell proliferation and migration; PIK3R1-FOXO3a-cMYC pathway; cell invasiveness; miR-155/TSPAN5 signaling axis	Kong *et al.*, 2008; Zhang *et al.*, 2018; Kia *et al.*, 2019; Kim *et al.*, 2018; Brown *et al.*, 2018; Yang *et al.*, 2020
miR-373/520c	*CD44, IL-8, ESR1, TXNIP, RABEP1, RELA, TGFBR2*	Oncogene-induced p53 pathway; metastasis; EMT process; pro-metastatic	Tang *et al.*, 2017; Eichelser *et al.*, 2014; Huang *et al.*, 2008; Yan *et al.*, 2011; Keklikoglou *et al.*, 2012
miR-9	*LIFR, E-cadherin, PTEN, DUSP14, FOXO1, AR, ESR1, STARD13, SOCS3, PIAS3, ONECUT2*	Cellular differentiation; Metastasis; cell proliferation, migration, and invasion; oestrogen regulated pathway; JAK/STAT signaling pathway; androgen receptor pathway; blockhe EV miRNA–ONECUT2 axis	Ma *et al.*, 2010; Chen *et al.*, 2012; Kia *et al.*, 2019; Liu *et al.*, 2017; Moazzeni *et al.*, 2017; Barbano *et al.*, 2017; Li *et al.*, 2020; Jiang *et al.*, 2020; Bandini *et al.*, 2020; Shen *et al.*, 2019
miR-10b	*RHOC, HOXD10, MAPRE1, PIEZO1, SRSF1, TP53, CDKN2A, TRA2B, FUT8, NR4A3, TBX5, PTEN, NF1, KLF4*	Metastasis; cell proliferation and invasion; AKT signaling pathway; transcriptional misregulation; pro-metastatic; EMT process; adhesion and migration	Ma *et al.*, 2007; Ma *et al.*, 2010; Meerson *et al.*, 2019; Guo *et al.*, 2018; M'hamed *et al.*, 2017; Kim *et al.*, 2018; Kim *et al.*, 2016; Negrini and Calin, 2008
miR-196a	*ANXA1, SPRED1, annexin-A1*	Cell proliferation; estrogen signaling	Luthra *et al.*, 2008; Jiang *et al.*, 2018; Rahim *et al.*, 2019
miR-221/222	*ERa, p27^{kip1}, p57, PTPμ, PTEN, Bim, TIMP3, SOCS3, Notch3, A20 (TNFAIP3), c-Rel, CTGF, GAS5*	Signaling pathways (Wnt, TGF-β, p53, MAPK, Notch, Erb B and Jak-STAT); focal adhesion; Wnt/β-catenin signaling; A20/c-Rel/CTGF signaling; Akt/NF-κB/COX-2 pathway; promotes EMT process; anti-apoptosis; cell proliferation	Rao *et al.*, 2011; Garofalo *et al.*, 2012; Liang *et al.*, 2018; Li *et al.*, 2017; Liu *et al.*, 2018; Santolla *et al.*, 2018; Zong *et al.*, 2019; Chen *et al.*, 2020; Tang *et al.*, 2019
Cluster 17/20 (miR-17-3p, miR-17-5p, miR-18, miR-19a, miR-19b, miR-20, and miR-92)	*AIB1, cyclin D1, STAT3, FOSL (Fra-1), IMPDH1, NPEPL1, PTEN, BRCA2, VEZF1*	Cell proliferation by Wnt, β-Catenin; LEF1 pathway; apoptosis; pro-metastatic; pro-angiogenesis	Hossain *et al.*, 2006; He *et al.*, 2005; Mu *et al.*, 2019; Liao *et al.*, 2017; Yang *et al.*, 2013
Tumor Suppressor miRNAs			
miR-206	*ESR1, NAMPT, MKL1, IL11, TWF1, SRF, WDR1, MRTF-A, neurokinin-1, WBP2*	ER signaling; inhibition of stemness and metastasis; cell migration and progression; modulation of tamoxifen sensitivity	Iorio *et al.*, 2005; Adams *et al.*, 2007; Samaeekia *et al.*, 2017; Xiang *et al.*, 2017; Zhou *et al.*, 2019; Hesari *et al.*, 2018; Ren *et al.*, 2017
miR-125a, b	*HER2, HER3, KIAA1522, BAP1*	Anchorage-dependent growth; differentiation; cell proliferation, migration and invasion	Mattie *et al.*, 2006; Scott *et al.*, 2007; Iorio *et al.*, 2005; Li *et al.*, 2018; Yan *et al.*, 2018
miR-34a	*Bcl-2, SIRT1, CCND1, CDK6, E2F3, MYC, ErbB2, Notch1, C22ORF28*	DNA damage; cell proliferation; drug resistance; FOXM1/eEF2K signaling axis	Li *et al.*, 2013; Christoffersen *et al.*, 2010; Welch *et al.*, 2007; Sun *et al.*, 2008; Wang *et al.*, 2017; Li *et al.*, 2017; Bayraktar *et al.*, 2018; Lin *et al.*, 2017; Rui *et al.*, 2018
miR-200	*BMI-1, ZEB1, ZEB2, QKI, MYB*	TGF-β signaling; EMT process; tamoxifen resistance	Uhlmann *et al.*, 2010; Cochrane *et al.*, 2010; Gregory *et al.*, 2008; Dykxhoorn *et al.*, 2009; Shimono *et al.*, 2009; Kim *et al.*, 2019; Gao *et al.*, 2019

(Continued)

Table 1 (continued).

miRNA	Target Genes	Role	References
miR-17-5p	*AIB1, CCND1, E2F1, STAT3*	Proliferation; apoptosis	Hossain *et al*., 2006; Yu *et al*., 2008; Liao *et al*., 2017
miR-31	*FZD3, ITGA5, M-RIP, MMP16, RDX, RHOA, Dkk1*	Metastasis; Wnt/β-catenin	Valastyan *et al*., 2009; Lv *et al*., 2017
let-7	*RAS, HMGA-2, H-RAS, LIN28, PEBP1, MCY, CCND1, CYP19A1, MAGE-A1, ADRB2, HIFIAN, CLDN12, MZF1*	Proliferation, differentiation; EMT pathway; EMC receptor interaction; pro-metastatic; angiogenesis; migration and invasion	Yu *et al*., 2007; Mayr *et al*., 2007; Wu *et al*., 2006; Johnson *et al*., 2005; Song *et al*., 2020; Qattan *et al*., 2017; Shibahara *et al*., 2012; Mi *et al*., 2019; Du *et al*., 2019; Tvingsholm *et al*., 2018
miR-205	*HER3, RunX2, HMGB1-RAGE, S1PR1*	P13K/Akt pathway; regulates the activity of $CD44^{+}/CD24^{-}$ breast cancer stem cells; EMT process and cell invasion	Iorio *et al*., 2009; Wang *et al*., 2019; Fang *et al*., 2020
miR-7	*PaK-1, FAK, PAK1, EGFR, KLF4, HoxB3, SET8, BRCA1, LASP1, RELA, MRP1, BCL2*	Anchorage independence; invasiveness; motility; inhibition of ESAM expression; reverses chemotherapy resistance	Reddy *et al*., 2008; Zhao *et al*., 2015; Moazzeni *et al*., 2017; Li *et al*., 2020; Hong *et al*., 2019
miR-30a	*SNAI1, Vimentin, Notch1, Snail, ROR1, Slug, TWIF1, vimentin, UBE3C*	Motility and invasiveness; EMT and metastasis; inhibition of cell viability and migration; apoptosis	Cheng *et al*., 2012; Wang *et al*., 2018; Kawaguchi *et al*., 2017; Zhang *et al*., 2017; Xiao *et al*., 2019

Dicer is a RNase III class of enzyme that cleaves the hair pin region of pre-miRNA to produce 20–30 nts of duplex miRNA composed of two single guide (5' to 3') and passenger (3' to 5') strands (Kobayashi and Tomari, 2016; Höck and Meister, 2008; Petri and Klinge, 2020). This guide strand joins the PAZ domain of RNA-induced silencing complex (RISC) to mediate post-transcriptional gene silencing of the target mRNA through Argonaute 2 (*AGO2*, RNase, catalytic part of RISC having "slicer" activity) which cleaves the target mRNA and the PIWI domain of RISC is responsible for the degradation of passenger strand (Höck and Meister, 2008; MacFarlane and Murphy, 2010; Yu and Cheah, 2017).

miRNA-induced silencing complex (miRISC, composed of *AGO2* and guide strand) mediate the degradation of target mRNA. miRISC follows Ago-catalyzed, decapping, deadenylation, and exonucleolytic mechanisms (Eulalio *et al*., 2008; Behm-Ansmant *et al*., 2006; Wahid *et al*., 2010). Once miRNA and target mRNA complement each other, AGO2's endonuclease activity is activated, cleaving the target mRNA (MacFarlane and Murphy, 2010). The miRISC inhibits the translation by obstructing the eIF4F complex, and mRNA circularization process of the target mRNA (Wahid *et al*., 2010). The GW182 family proteins bind to *AGO2* are recruited by miRISC and act as a scaffold for the complex poly(A)-deadenylase PAN2-PAN-3 and CCR4-NOT proteins (MacFarlane and Murphy, 2010; O'Brien *et al*., 2018). The poly(A)-deadenylation process is initiated by the PAN2-PAN3 complex and completed by the CCR4-NOT protein complex. This process is further enhanced through the interaction of poly(A)-binding protein (PABPC) and GW182 tryptophan (W)-repeats (MacFarlane and Murphy, 2010; O'Brien *et al*., 2018). The exosome (with 3'-5' exonuclease activity) mediates the degradation process and the enzymes, decapping protein 1 (Dcp 1) and decapping protein 2 (Dcp 2) facilitate the process by inducing exoribonuclease 1 (XRN1), which target 5'-3' mRNA degradation (MacFarlane and Murphy, 2010; O'Brien *et al*., 2018).

Identifying miRNA in breast cancer cells

miRNAs have crucial role in gene regulation, so it is becoming imperative to develop and improve methods that can detect miRNAs because their detection in breast cancer is difficult due to their small size, low abundance, high level of sequence similarities (Chandrasekaran *et al*., 2019; Ye *et al*., 2019). The most well-known traditional methods for the detection of miRNA are microarray, *in situ* hybridization, bead-based flow cytometry, next-generation sequencing (NGS), northern blotting, and quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Ye *et al*., 2019; van Schooneveld *et al*., 2015). Out of these methods, northern blotting and *in situ* hybridization are low throughput methods, whereas microarray, bead-based flow cytometry, qRT-PCR and NGS are high throughput methods (van Schooneveld *et al*., 2015).

Northern blotting is the most standardized and highly specific method that can detect mature miRNA and their precursors (Ye *et al*., 2019; Várallyay *et al*., 2008). Besides these, the northern blotting method has a poor sensitivity and cannot detect the RNAs present in low amounts in few cells (Ye *et al*., 2019). qRT-PCR method is a highly sensitive and specific method used for evaluating miRNA and for the authentication of data prevailed from other detection platforms (Balcells *et al*., 2011; Yu and Cheah, 2017). The bead-based flow cytometry method is moderate in sensitivity and strong in specificity, whereas the *in situ* hybridization method is low in sensitivity and specificity (van Schooneveld *et al*., 2015). Microarray is a rapid method that can examine plenty of miRNAs from vast numbers of samples, with low

sensitivity and specificity (Li and Ruan, 2009; Cissell and Deo, 2009). All these methods recognize only known miRNA structures, but NGS is the latest high sensitivity and specificity approach that enables new miRNAs to be recognized (van Schooneveld *et al.*, 2015; Creighton *et al.*, 2009). In the case of breast cancer, the upregulated levels of miR-21 can also be detected from tissues, blood, and serum (Yu and Cheah, 2017; Savad *et al.*, 2012).

Oncogenic role of miR-21 in breast cancer cell proliferation and metastasis

The "oncomiR" *miR-21* is believed to be involved in tumor proliferation and metastasis in breast tissues by controlling the expression of tumor suppressor genes such as *programmed cell death 4* (*PDCD4*), *tropomyosin-1* (*TPM1*), *phosphatase and tensin homolog* (*PTEN*), *mammary serine protease inhibitor* (*MASPIN*); an apoptosis suppressor gene, *B-cell lymphoma 2* (*Bcl-2*), and leucine zipper transcription factor-like 1 gene [(*LZTFL1*), a tumor suppressor] (Si *et al.*, 2007; Zhu *et al.*, 2007; Frankel *et al.*, 2008; Qi *et al.*, 2009; Zhu *et al.*, 2008; Wei *et al.*, 2010; Wang *et al.*, 2019) (illustrated in Fig. 2). These genes play a critical role in breast cancer and are targeted by miR-21.

Programmed Cell Death 4 (PDCD4)

In both immune/non-immune cells, the programmed cell death 4 gene is a tumour suppressor (Jiang *et al.*, 2017). The PDCD4 protein contains three domains (two MA-3 domains and the N-terminal domain) that play a role in transcription and translation inhibition (Jiang *et al.*, 2017). Owing to the binding of its MA-3 domains to eIF4A, PDCD4 prevents the translation of mRNAs having structured 5'UTRs, hindering eIF4A binding to eIF4G, even blocking eIF4A's helicase activity, thus repressing the translation initiation mechanism of many genes, such as *p53*, *Atg5*, *pro-caspase 3*, and *LXR-α* (Kroczynska *et al.*, 2012; Yang *et al.*, 2004). By interacting with its N-terminal domain with A-myb & c-myb (RNA secondary structures), PDCD4 represses the elongation mechanism of translation (Singh *et al.*, 2011). PDCD4 prevents tumor cell proliferation and invasion via inducing their apoptosis, leading to its upregulation in various tumors like breast, ovarian, gastric, esophageal, lung, hepatocellular, colon and glioma tumors (Powers *et al.*, 2011; Wei *et al.*, 2012; Jiang *et al.*, 2017). The protein arginine methyltransferase 5 (PRMT5) deregulates the activity of the PDCD4 gene, resulting in breast cancer cell proliferation and invasiveness (Powers *et al.*, 2011). miR-21 has been shown to target PDCD4 in tumour cells, thereby downregulating its expression (Sheedy *et al.*, 2010).

PDCD4 is a target of miR-21 for breast cancer (Frankel *et al.*, 2008). The miR-21 binding sites in the PDCD4 3'UTR are shown in Fig. 3A, which shows how miR-21 interacts with the PDCD4 3'UTR. To assess the fact that miR-21 specifically targets PDCD4, the firefly luciferase reporter assay was carried out. Frankel and his colleagues first cloned PDCD4's 400–500 bp of 3'UTR into the pGL3 vector and then developed single (pGL3-PDCD4MUT1) and double (pGL3-PDCD4MUT2) mutations in the pGL3-PDCD4 seed region. PGL3-PDCD4, pGL3-PDCD4MUT1 & pGL3-PDCD4MUT2 were transfected into HEK293 cells and luciferase activity was tested after 24 h.

Results showed that miR-21 had no effect on pGL3-PDCD4MUT2 regulation but had little effect on pGL3-PDCD4MUT1 regulation (as shown in Fig. 3B), leading to the inference that there is a clear association between miR-21 and PDCD4 seed region 3'UTR. To verify the effects of miR-21 inhibition, they knocked down PDCD4 in MCF-7 cells via siRNA and found that PDCD4 siRNA mediated the antiproliferative effect on MCF-7 cells (see Figs. 3C, D). Thus, revealing the significance of PDCD4 by miR-21 as a putative target for breast cancer (Frankel *et al.*, 2008).

Another research by Zhu *et al.* (2008) showed that miR-21 interacted with PDCD4's 3'UTR, resulting in its reduced expression in MDA-MB-231 cells. Anti-miR-21 also led to increased PDCD4 expression, indicating the role of miR-21 in breast cancer cell invasion by directly interacting with the PDCD4 target gene (Zhu *et al.*, 2008).

Tropomyosin-1 (TPM1)

Tropomyosin-1, also known as *TM1* and the actin-binding cytoskeletal protein, is a TPM family isoform that functions as a tumour suppressor gene by preventing cancer cell invasion by persuading apoptosis (Wang *et al.*, 2015; Pan *et al.*, 2017; Qi *et al.*, 2009; Wang *et al.*, 2019). Downregulation of TPM1 has been observed in breast cancer, glioma cancer, renal cell carcinoma, human oral squamous cell carcinoma (OSCC), and cholangiocarcinoma (Wang *et al.*, 2015; Dube *et al.*, 2015; Pan *et al.*, 2017; Bharadwaj and Prasad, 2002; Yang *et al.*, 2013; Ku *et al.*, 2010). The expression of TPM1 in the OSCC is regulated by miR-21 (Pan *et al.*, 2017). In addition, Zhu *et al.* (2007) in their report, showed that TPM1 is targeted by miR-21 in breast cancer to inhibit the TPM1 mediated apoptosis of cancer cells. They conducted two-dimensional differentiation in-gel electrophoresis, where they found the upregulated TPM1 expression in tumors procured from MCF-7 cells by antisense miR-21 oligonucleotides (anti-miR-21). miR-21 controls TPM1 expression by base-pairing with the V1 variant 3'UTR of TPM1, including miR-21's putative binding sites. For further evidence, the V1-3'UTR was cloned into a luciferase reporter. Luciferase assay results suggested that anti-miR-21 increased Luc-TPM1-V1-UTR activity, while miR-21 decreased its activity. Moreover, the effect of miR-21 inhibition on TPM1 was also tested at the level of translation. MTT assay revealed that TPM1-V1 repressed the growth of MCF-7 cells *in vitro*, and soft agar assay results also showed that TPM1-V1 reduced colony formation leading to anchorage-independent growth repression. Altogether, we can conclude that miR-21 uses TPM1 to control its expression in MCF-7 cells as a novel target.

Phosphatase and tensin homolog (PTEN)

PTEN on chromosome 10 is recognized as a tumor suppressor gene coding 403 amino acids protein, which functions as a metabolic regulator of a number of cellular processes like glycogen synthesis, lipid and mitochondrial metabolism, glycolysis, and gluconeogenesis (Chen *et al.*, 2018). It has dephosphorylation activities for both proteins and lipids due to which it is known as dual phosphatase enzyme (Chu and Tarnawski, 2004). PTEN has five domains: 1) N-terminal domain, responsible for the binding of phosphatidylinositol phosphate (PIP) substrates; 2) Phosphatase domain, carries

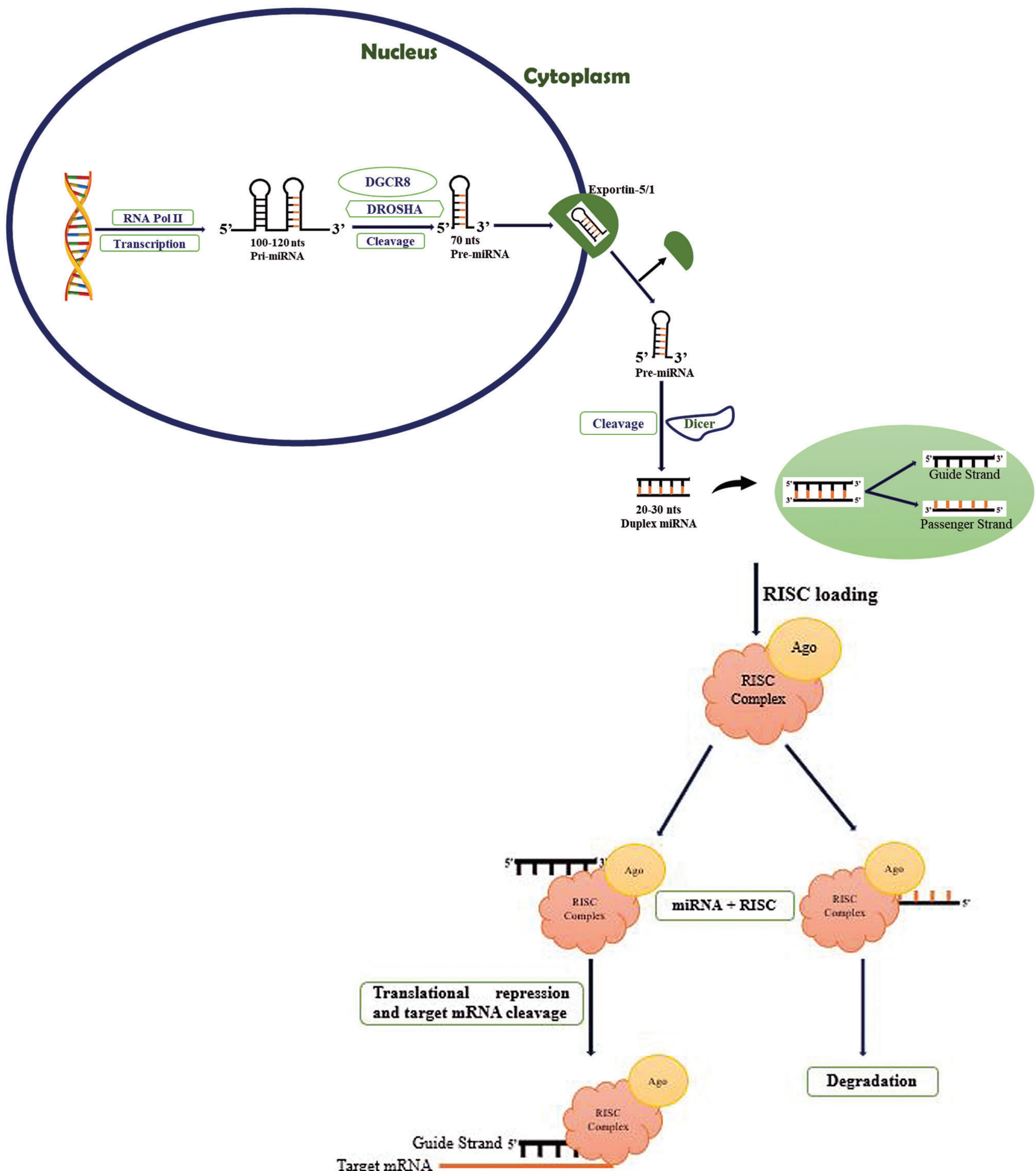

FIGURE 1. Biosynthesis of miRNA. RNA polymerase II is responsible for the transcription of 100–120 nts pri-miRNA in the nucleus. The pri-miRNA is trimmed into 70 nts pre-miRNA by DROSHA and DGCR8, which is then exported by exportin 5/1 into the cytoplasm, where it is further processed by Dicer enzyme forming 20–30 nts duplex miRNA with the guide (5' to 3') and passenger (3' to 5') strands. The guide strand interacts with the PAZ domain of the RISC, causing Argonaute 2 (*AGO2*) to cause post-transcriptional gene silencing and cleavage of the target mRNA, while the RISC's PIWI domain degrades the passenger strand.

acetylation sites and CX5R signature motif for the enzymatic actions; 3) C2 domain, a regulatory domain which contains a phospholipid-binding site and known for PTEN cell localization; 4) C-tail, which preserves phospho-sites and is responsible for the stability of protein; 5) PDZ domain, which is not well known (as shown in Fig. 4) (Chu and Tarnawski, 2004; Jerde, 2015; Chen *et al.*, 2018). PTEN has regulatory functions in several processes, including cell migration, cell cycle arrest, MAP kinase signaling and angiogenesis (Chu and Tarnawski, 2004). Any mutations in PTEN gene lead to the progression of sporadic breast cancer, renal cell carcinoma, ovarian cancer, lung cancer, thyroid cancer, lymphoma cancer, hepatocellular carcinoma, lymphoma, head & neck cancer, prostate cancer, and glioblastoma (Chu and Tarnawski, 2004; Meng *et al.*, 2007; Halachmi *et al.*, 1998; Shao *et al.*, 1998; Forgacs *et al.*, 1998;

microRNA-21

Target genes

Programmed cell death 4 (*PDCD4*)

Tropomyosin -1 (*TPM1*)

Phosphatase and tensin homolog (*PTEN*)

Mammary serine protease inhibitor (*MASPIN*)

Leucine zipper transcription factor-like 1 (*LZTFL1*)

B-cell lymphoma 2 (*Bcl-2*)

Tumor suppressor genes, prevent the proliferation and invasion of tumor cells via inducing the apoptosis

Tumor suppressor, regulates β-catenin nuclear signaling & EMT process

Anti-apoptotic gene, stimulates cell proliferation by restricting the functions of Bak & Bax proteins

FIGURE 2. Depicts miR-21's target genes and their functions in tumorigenesis.

A

GUGGAAUAU---UCUAAUAAGCUA Position 224-248 of *PDCD4* 3'UTR

AGUUGUAGUCAGACUAUUCGAU *hsa-miR-21*

B

Luciferase/Renilla ratio relative to control

pGL3 PDCD4; pGL3 PDCD4 + miR-21; pGL3 PDCD4 + lin-4; pGL3 PDCD4MUT1; pGL3 PDCD4MUT1 + miR-21; pGL3 PDCD4MUT1 + lin-4; pGL3 PDCD4MUT2; pGL3 PDCD4MUT2 + miR-21; pGL3 PDCD4MUT2 + lin-4

C

PDCD4 siRNA; Control siRNA

PDCD4

Vinculin

D

p < 0.003

% Viable cells

Control siRNA; PDCD4 siRNA

LNAscramble; LNAmiR-21

FIGURE 3. Represents that PDCD4 is a putative target of miR-21. A) shows the interaction of miR-21 with the 3'UTR of PDCD4. B) shows firefly luciferase reporter assay results, where co-transfection of HEK cells was done with pGL3-PDCD4, pGL3-PDCD4MUT1, pGL3-PDCD4MUT2 along with a Renilla luciferase transfection control plasmid, either alone or together with miR-21/lin-4 precursor miRNAs. Shown are relative luciferase values normalized to transfections without miRNA. Data are shown as the mean S.D. of four replicates and are representative of two independent experiments. ***, p_0.001 using a two-tailed *t* test. C) shows western blotting results where siRNA mediated knockdown of PDCD4 was shown in MCF-7 cells. D) shows the growth assay of PDCD4/control siRNA treated 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in MCF-7 cells and transfection with inhibitors against miR-21/a scrambled control. Quantification of cell number was done after 5 days of transfection. Data are shown as the mean _ S.D. of three replicates and are representative of three independent experiments. The *p* value was calculated using a two-tailed *t* test (Frankel *et al.*, 2008).

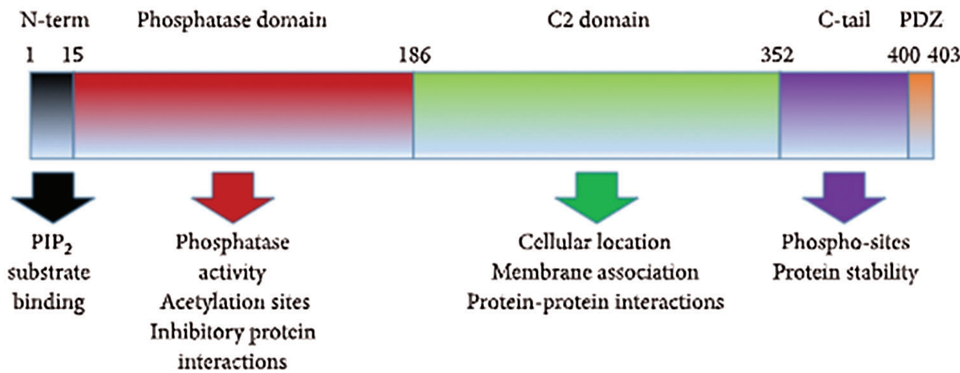

FIGURE 4. Protein domains of PTEN (Jerde, 2015).

Saito *et al.*, 2000). In human hepatocellular cancer, miR-21 is known to regulate the *PTEN* expression and pathways which are mediated by *PTEN* (Meng *et al.*, 2007).

A research conducted by Gong *et al.* (2014) showed that miR-21 decreases PTEN expression in tumors of breast phyllodes. Like PDCD4 and TPM1, PTEN also has miR-21 binding sequences at 3'UTR (Zhu *et al.*, 2007; Frankel *et al.*, 2008; Gong *et al.*, 2014). PTEN protein levels were increased via miR-21 antisense oligonucleotides while, decreased via miR-21 mimics in stromal fibroblasts, demonstrating the role of miR-21 in targeting PTEN to increase the proliferation of phyllodes tumors cells (Gong *et al.*, 2014). PTEN is known for the regulation of fibroblast activation protein (FAP) function (FAP is familiar as a serine protease and myofibroblast marker to increase the proliferation of phyllodes tumors) (Liu *et al.*, 2012; Gong *et al.*, 2014). The PTEN knockdown raised the protein expression levels of FAP during the myofibroblast differentiation process, suggesting the role of PTEN in proliferation. Moreover, reintroduction of PTEN resulted in a decrease in both PTEN mRNA and protein levels in benign tumors, thus inhibiting the proliferation of tumor cells by miR-21. Together, these findings concluded that miR-21 downregulates the *PTEN* expression to induce cell proliferation by increasing FAP expression in breast phyllodes tumor (Gong *et al.*, 2014).

Mammary Serine Protease Inhibitor (MASPIN)

Mammary serine protease inhibitor is related to the serpin family (serine protease inhibitor family) (Zhang, 2002). Due to its ability to prevent tumor invasion by inducing apoptosis in tumor cells found on the 18q21.3-q23 chromosome, it is also recognized as a tumor suppressor gene (Berardi *et al.*, 2013; Zhang, 2002). It is also known to prevent the process of angiogenesis (Zhang, 2002). Owing to its nuclear/cytoplasmic location in cancer cells, its expression is downregulated in various cancers like breast, gastric, prostate, melanoma and upregulated in gallbladder, thyroid, colorectal, pancreatic cancers (Berardi *et al.*, 2013).

Maspin decreased MDA-MB-231 tumour cell invasion in the case of breast cancer (Zhu *et al.*, 2008). With Flag-tagged maspin, they transfected MDA-MB-231 cells and confirmed the invasiveness of tumour cells through western blotting. However, by targeting it, the oncomir miR-21 downregulated maspin expression in MDA-MB-231 cells. Maspin also has 3'UTR binding sites for miR-21, so they cloned 3'UTR of maspin into pGL3 vector (luciferase gene) to check the Luc-Maspin-3'UTR luciferase activity. They found that miR-21 repression of luciferase activity was greater than 40%. Furthermore, anti-miR-21 increased the maspin expression, leading to the conclusion that by targeting its 3'UTR, miR-21 inhibits the maspin expression in MDA-MB-231 cells (Zhu *et al.*, 2008).

B-cell Lymphoma 2 gene (Bcl-2)

B-cell lymphoma 2 is a 26 kDa protein present on human chromosome 18q21 with a hydrophobic carboxyl terminus positioned on the outer mitochondrial membrane and an anti-apoptotic gene as well (Lu *et al.*, 1996). It suppresses

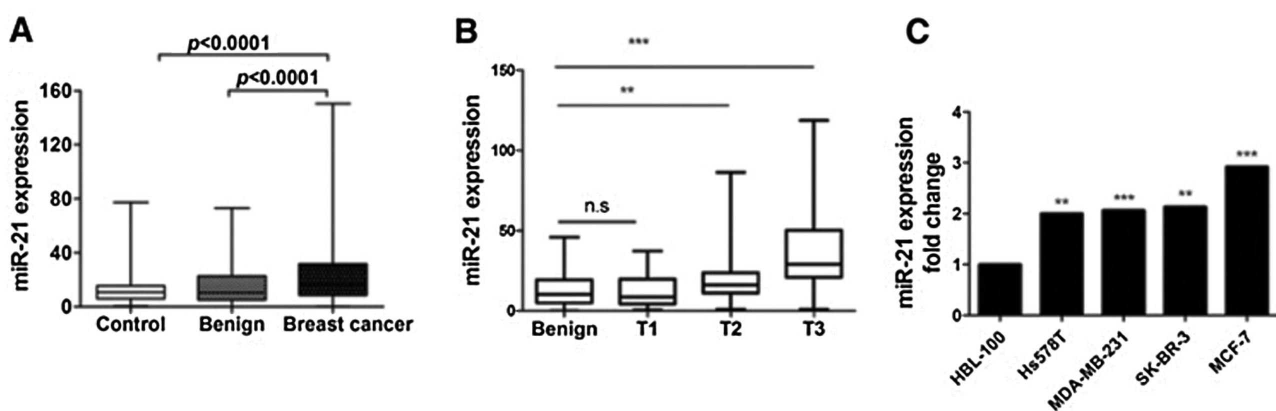

FIGURE 5. The levels of miR-21 in breast cancer patient plasma and cell lines. (A) Plasma miR-21 levels in 252 breast cancer patients, 127 healthy controls, and 82 benign breast cancer patients ($p < 0.0001$). (B) Plasma miR-21 levels in luminal A, luminal B, Her-2+ and basal-like types of breast cancer patients ($p < 0.05$). (C) The mRNA levels of miR-21 in HBL-100, Hs578T, MDA-MB-231, SK-BR3, and MCF-7 cell lines (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) (Wang *et al.*, 2019).

LZTFL1 3'-UTR 341-347 nt 5'...UUAAUAAAUGUUAAAUAAGCUAACAA...3'

hsa-miR-21 3'...AGUUGUAGUCAGAC UAUUCGAU...5'

LZTFL1 3'-UTR mutant 5'...UUAAUAAAUGUUAA GUAGCGCAACAA...3'

FIGURE 6. The predicted binding site of miR-21 in the 3'-UTR of wild type and mutant LZTFL1 (Wang *et al.*, 2019).

the caspase-mediated cell death and stimulates cell proliferation by restricting the function of pro-apoptotic proteins, Bak and Bax (Lu *et al.*, 1996; Wickramasinghe *et al.*, 2009). Upregulated levels of bcl-2 gene are found in many cancers like lymphoma, breast, colorectal, thyroid, and cervical (Flangea *et al.*, 2008; Manne *et al.*, 2000; Zhou and Wang, 2015). Research on human glioblastoma U87MG cells revealed that miR-21 overexpression resulted in decreased Bax expression and increased bcl-2 gene expression along with reduced caspase-3 activity (Shi *et al.*, 2010). Similarly, miR-21 controls tumorigenesis by upregulating the expression of the bcl-2 gene in breast cancer as miR-21 was highly overexpressed in breast tumors compared to the matched normal breast tissues 157 human miRNAs analysed. (Si *et al.*, 2007). To investigate the function of miR-21, MCF-7 cells were transfected with anti-miR-21 oligonucleotide.

They showed that anti-miR-21 inhibits *in vitro* cell growth and tumor growth in the xenograft mouse model. Furthermore, to assess anti-miR-21 induced apoptosis, transfected cells were given Z-VAD-fmk, a caspase inhibitor, to counteract growth inhibition mediated by anti-miR-21. Apoptosis was caused by reduced Bcl-2 protein expression in anti-miR-21 transfected MCF-7 cells. These findings concluded that miR-21 should be used as a therapeutic target for breast cancer because of its essential function in controlling the Bcl-2 gene (Si *et al.*, 2007). In another study, breast cancer cell lines were developed, which are paclitaxel-resistant, and it was shown that down regulation of miR-21 via its mimic enhance the sensitivity against paclitaxel in the developed cell lines and increase the expression of Bcl-2 (Zhao *et al.*, 2015).

Leucine Zipper Transcription Factor-like 1 (LZTFL1)

The 3p21.3 region of the chromosome contains the leucine zipper transcription factor-like 1 (LZTFL-1) gene expressed in epithelial cells of a diversity of normal cells (Wei *et al.*, 2010). LZTFL-1, a cytoplasmic protein, regulates β-catenin nuclear signaling, ciliary protein trafficking, and the epithelial-mesenchymal transition (EMT) process (Wei *et al.*, 2016). It represses EMT by inhibiting the mitogen-activated protein kinase (MAPK) signaling pathway, which is especially important in lung cancer (Wei *et al.*, 2010; Wei *et al.*, 2019). It functions as a tumor suppressor by stabilizing E-cadherin-mediated adherens junctions and inhibiting β-catenin relocation into the nucleus, preventing cell invasion and EMT-mediated breast cancer metastasis (Wang *et al.*, 2014; Wang *et al.*, 2019). Wang *et al.* (2019) discovered that *LZTFL1* could be used as a new target in breast cancer cells via miR-21, facilitating cell invasion and metastasis. miR-21 was found to be upregulated in the plasma of three patient groups and various cell lines.

The level of plasma miR-21 was significantly higher in the breast cancer patient group than the benign breast cancer patient group and healthy control group (see Fig. 5A), suggesting that plasma miR-21 level may be used as a potential diagnostic biomarker for breast cancer. The histopathological examination showed an upregulated level of miR-21 in breast cancer types Her-2+ and luminal B (as shown in Fig. 5B), and *in vitro* analysis results showed miR-21 upregulate in all cell lines of breast cancer (MDA-MB-231, Hs578T, MCF-7, and SK-BR3) compared to immortalized mammary epithelial (HBL-100) cell line (see Fig. 5C). As demonstrated by luciferase reporter assay, miR-21 directly targets *LZTFL1*, where miR-21 overexpression resulted in decreased luciferase activity in HEK293T cells transfected with luciferase gene containing either wild-type or mutant 3'UTR of LZTFL1 (see Fig. 6).

The miR-21/LZTFL1/-catenin axis induces the EMT process. The effect of miR-21 inhibition and LZTFL1 knockdown on various EMT markers such as N-cadherin, vimentin, E-cadherin and claudin-1. miR-21 inhibition decreased N-cadherin and vimentin expression while increasing E-cadherin and claudin-1 expression, whereas LZTFL1 knockdown reversed the expression of these markers (as shown in Fig. 7). Immunofluorescence assay

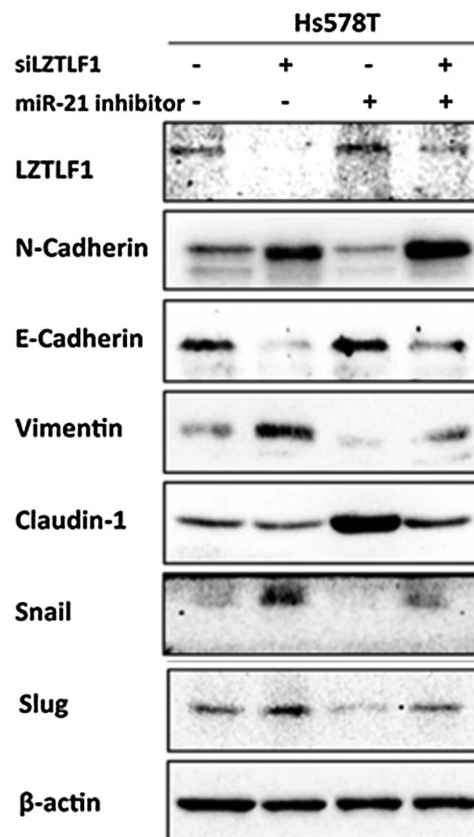

FIGURE 7. miR-21/LZTFL1 regulates β-catenin nuclear translocation and EMT process. The protein levels of EMT markers in Hs578T cells treated with miR-21 inhibitor, LZTFL1 siRNA alone, or combined for 48 h (Wang *et al.*, 2019).

revealed that translocation of β-catenin in the nucleus was increased by miR-21 which contributed to the transcription of snail and slug transcription factors (EMT markers) (as shown in Fig. 7), while LZTFL1 repressed it. Therefore, miR-21 controls EMT by suppressing the LZTFL1, which increases β-catenin nuclear translocation, resulting in breast cancer proliferation and metastasis. This was also proved in BALB/c nude mice inoculated with Hs578T (human breast cancer) cells. miR-21 overexpression increased tumor size, weight, and volume (see Figs. 8A–8C) as well as lymph node invasion (see Figs. 8D and 8E). Furthermore, overexpression of miR-21 increased metastasis in liver and lung tissues (as shown in Figs. 8F and 8G) (Wang *et al.*, 2019).

Summary

There is an extensive role of 'oncomiR' miR-21 in various stages breast cancer viz invasion, development, and metastasis. Different studies have demonstrated miR-21 upregulation in breast cancer cells by targeting multiple genes involved in breast cancer tumorigenesis. As mentioned above, miR-21 overexpression, through the downregulation of tumour suppressor genes, increases cell proliferation (Frankel *et al.*, 2008; Zhu *et al.*, 2007; Gong *et al.*, 2014). miR-21 specifically interacts with PDCD4, TPM1, PTEN, and maspin 3'UTR, contributing to the suppression of apoptotic activities (Frankel *et al.*, 2008; Zhu *et al.*, 2007; Zhu *et al.*, 2008; Gong *et al.*, 2014). It also promotes breast cancer cell proliferation by increasing the bcl-2 gene expression. These studies indicate that alteration in the expression of miR-21 can be a beneficial intervention in the treatment of breast cancer. Various molecular biology-based techniques have been developed to detect the alteration in the expression of miR-21. Besides this, biosensor-based detection of miR-21 is a new area of research that could be more accessible and can help in point-of-care testing (Wang *et al.*, 2020; Meng *et al.*, 2020; Sun *et al.*, 2018). Another sensitive tool to detect miR-21 in breast cancer is DNA–Peptide dendrimer and mass spectrometric method. It would also be essential to understand the role of miRNA in connection with the changing microbiome, which will help in early diagnosis and be used as a therapeutic biomarker (Allegra *et al.*, 2020; Rastogi *et al.*, 2020; Kashyap *et al.*, 2021).

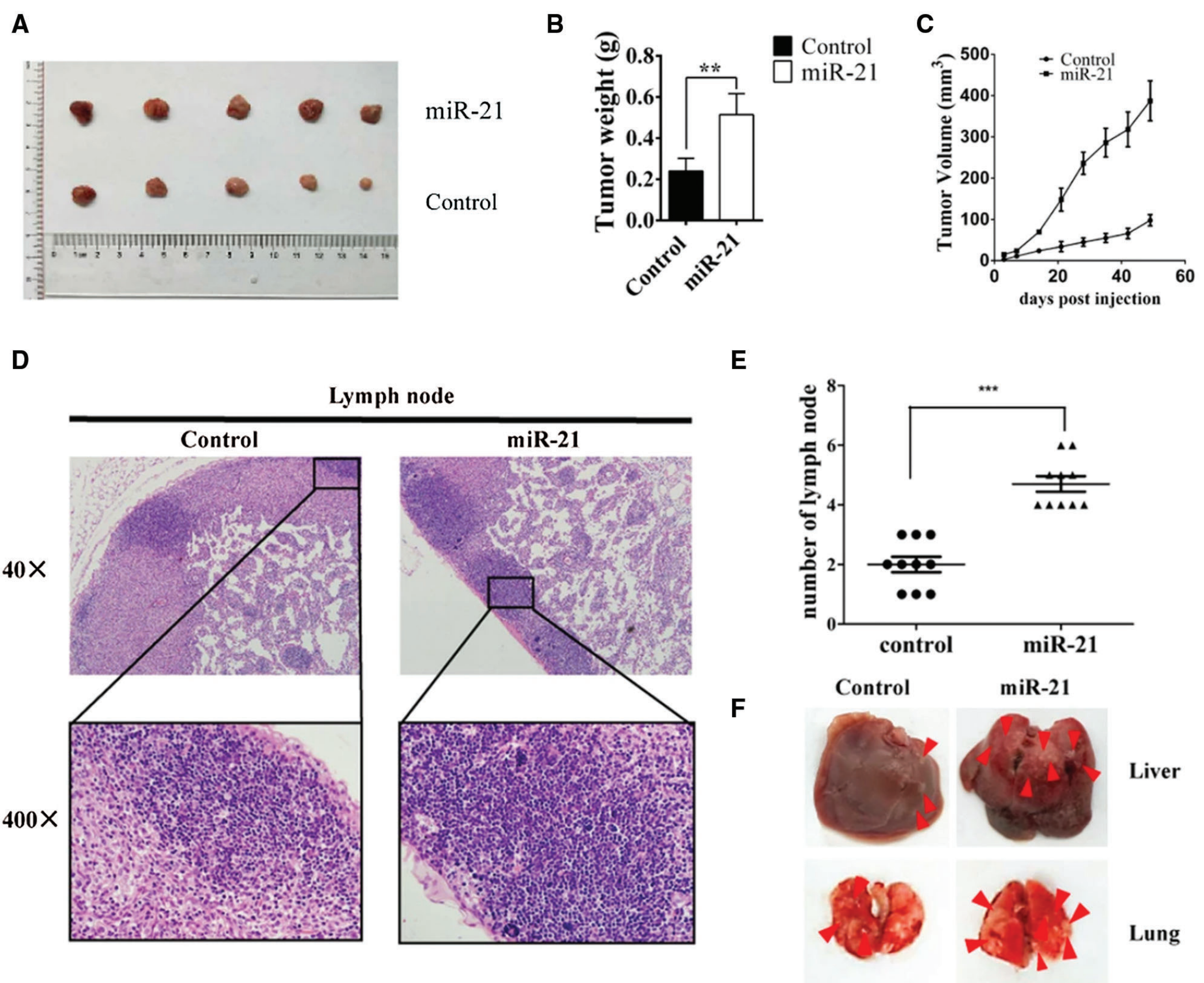

FIGURE 8. miR-21 promotes breast cancer proliferation and metastasis *in vivo*. (A) Xenografted tumors were obtained from miR-21-treated Hs578T and control Hs578T cells *in situ*. (B and C) Tumor weight and volume were observed and recorded in the groups indicated above. (D and E) The number of lymph nodes invaded was determined. (F and G) Liver and lung tissues were obtained, and the metastatic cells were visualized (Wang *et al.*, 2019).

Acknowledgement: Authors acknowledge the support from Maharishi Markandeshwar (Deemed to be University) Mullana and Shoolini University, Solan.

Author Contribution: The authors confirm contribution to the paper as follows: study conception and design: AKS, RVS; data collection: PT, NKS, AC; draft manuscript preparation: PT, VKT, SSS. All authors compared different studies and approved the final version of the manuscript.

Funding Statement: Authors received no specific funding for this study.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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