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WWTR1-AS1 lncRNA as a Novel Potential Diagnostic Biomarker in Breast Cancer

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ABSTRACT

Breast cancer is the most lethal form of cancer in women, and patients face serious health risks. Long non-coding RNAs are involved in a variety of regulatory processes and can influence cancers development at different levels. This study aimed to introduce a central regulatory lncRNA based on differentially expressed proteins in breast cancer and evaluate its expression level in breast tissues. In this study, proteomic data was obtained from ProteomeXchange and then differentially expressed proteins were detected. The Enrichr database was used to identify the regulatory factors of differentially expressed proteins, and functional enrichment analysis was used to demonstrate key signaling pathways and biological processes. Eventually, a lncRNA with the highest rank in the central hub was chosen, and its expression level was measured by RT-qPCR in 15 breast cancer tissues and their adjacent nontumor tissues. Proteomic analysis recognized 1149 differential expressed proteins in breast cancer with regulatory agents consisting of 76 TFs, 61 kinases, 366 miRNAs, and 162 lncRNAs. A multi-regulatory network with 1811 nodes and 4022 edges was constructed based on differentially expressed proteins and their associated elements. In addition, these regulatory elements were related to three biological functions and 11 pathways. Finally, bioinformatic analysis identified lncRNA WWTR1-AS1 as having the highest node score involved in different mechanisms. Functional experiments confirmed that the expression level of the lncRNA WWTR1-AS1 was significantly increased in breast cancer patients. ROC analysis suggested that this lncRNA can be used as a reliable biomarker. Our data provide evidence that the lncRNA WWTR1-AS1 is an effective factor in the regulation of DEPs, is associated with malignant features in breast cancer, and might be useful as a prognostic marker in Breast cancer.

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Introduction

According to the Global Cancer Observatory (GCO) breast cancer is the most prevalent malignant tumor in women and the leading cause of cancer mortality in women reported globally 17.7% in 2022 (https://gco.iarc.fr/en). Although therapeutic approaches and diagnostic methods have been greatly enhanced, many patients still face serious health risks, including death and recurrence (Choi *et al.*, 2021). Although considerable research has revealed that there are

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important genes and proteins involved in the pathogenesis of breast cancer, the specific and certain mechanisms responsible for its development remain are still unknown. However, the evidence supported the significance of specific genes in breast cancer. For example, BRCA1-associated protein-1 (BAP1) is a therapeutic target in breast cancer (Qin *et al.*, 2015), and Golgi membrane protein 1 (GOLM1) regulates matrix metallopeptidase 3 (MMP13), which in turn promotes the aggressive progression of breast cancer cells (Zhang *et al.*, 2019a).

Long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) are non-coding regulatory molecules that can affect breast cancer development at different levels (Augoff *et al.*, 2012). LncRNAs longer than 200 nt are involved in a variety of regulatory processes, such as translation, chromatin remodeling, post-translational protein modification, cell signaling, and the regulation of transcription through interactions with proteins and RNAs. Moreover, dysregulation of lncRNAs has been linked to adverse effects on human health. There is growing confidence that lncRNA expression profiling can be used as a biomarker for the diagnosis of human diseases (Choudhuri, 2023). WW domain-containing transcription regulator 1 antisense RNA 1 (WWTR1-AS1) is a newly discovered lncRNA, located in the 3q25.1, which is considered a natural antisense transcript (NAT) via the connection with the untranslated region (5′--UTR) of WWTR1. Although recent evidence has shown that WWTR1-AS1 an oncogenic lncRNA that is overexpressed in head and neck squamous cell carcinoma (Li *et al.*, 2019a) and cervical squamous cell carcinoma (CSCC) (Zhou *et al.*, 2024), its status in breast cancer is unknown.

According to previous studies, analysis of genomics, transcriptomics, and proteomics data obtained from patients can guide the discovery of disease-related factors or associated markers. These elements are often dysregulated (increases or decreases) in patients compared with those in the normal state. This approach has helped researchers introduce several essential molecules related to disease progression (Civelek and Lusis 2014; Molendijk and Parker 2021). Hence, we re-analyzed a proteomics dataset of patients with breast cancer and detected differentially expressed proteins (DEPs), their associated upstream regulatory elements, transcription factors (TFs), long non-coding RNAs (lncRNAs), and validated miRNAs. Finally, among the top identified central lncRNAs, we evaluated lncRNA WWTR1-AS1 expression alteration experimentally.

Materials and Methods

Proteomics data acquisition and analysis

The expression profile of PXD012431 (a High-throughput mass spectrometry and bioinformatics analysis of breast cancer proteomic data) was obtained from ProteomeXchange, which contains 13 samples, including six primary breast tumors and seven normal breast tissues from healthy volunteers (Vizcaíno *et al.*, 2014; Gomig *et al.*, 2019). LFQ- Analyst, a web platform, was used to visualize label-free proteomics data that were preprocessed using MaxQuant (v1.5.5.1) and produced a downloadable FASTA file in UniProt (Tyanova *et al.*, 2016a). Trypsin was chosen as the digestive enzyme with up to two potentially missed cleavage sites. Both the peptide-to-spectrum match (PSM) and protein false discovery rate (FDR) were set to 1% for peptides and proteins. Carbamidomethylation (C) was selected as fixed modification, and oxidation of methionine (M) and acetylation (N-terminal of the protein) were used as variable modifications for the quantification of unmodified and modified peptides. Matching between run options was enabled. Mass analyzer parameters were set based on software default.

Differentially expression analysis

To detect differentially expressed proteins, a statistical analysis of MaxLFQ data was performed using Perseus and processed according to the workflow (Tyanova *et al.*, 2016b). First, to explore the quality of samples in the exploited dataset, principal component analysis (PCA), which is a hierarchical unsupervised technique, was used. The quantified proteins were then filtered in terms of reverse hits and contaminants that were only identified by site, and MaxLFQ values were transformed to log2. Proteins with more than three valid values were excluded from the

analysis. The student'' t-test was conducted for statistical analysis using permutation-based FDR to correct multiple hypothesis testing. The randomization number was set at 250. Proteins with FDR<0.01 were considered as DEPs.

Enrichment analysis

To provide a comprehensive understanding of breast cancer regulatory elements, the Enrichr database was used for transcription factor and kinase enrichment analysis (Kuleshov *et al.*, 2016). Additionally, the MetaScape database was used to identify all discovered DEPs and their expected regulatory components using gene ontology (GO) and pathway enrichment analyses (Zhou *et al.*, 2019). The REVIGO tool was used to summarize the GO terms to parent terms (Supek *et al.*, 2011). Furthermore, to detect validated miRNAs and lncRNAs that regulate DEPs TargetScan and lncHUB applications in Enrichr were used (Mon-López and Tejero-González 2019). Across all analysis steps, adj.pvalue <0.5 was confirmed to be a significant threshold for the statistical analysis. The CluePedia (v1.5.8) plugin in Cytoscape was used to create an integrative map with all recognized and predicted items (Shannon *et al.*, 2003; Bindea *et al.*, 2013). The STRING database was used to retrieve the all-interaction type including activation, inhibition, and post-translational modification (PTM) (Szklarczyk *et al.*, 2019). The confidence cut-off was set to 0.6. Then, miRNAs and lncRNAs- interactions were merged into the network. Eventually, a Cytoscape Network analyzer was used to assess the constructed merge network regarding topological parameters.

Clinical samples

We obtained tumor and adjacent normal samples from 30 patients with breast cancer who were undergoing treatment at the Milad Hospital in Isfahan, Iran. The samples were tested by a pathologist, and normal or cancerous status was confirmed. Ethics Committee guidelines were followed for sample collection, and all participants provided informed consent to be included in this study. Females who had affected breast cancer in grades I - III and were undergoing treatment at Milad Hospital in Isfahan were included in the study. Males who had affected breast cancer and female breast cancer patients with reoccurrence or no treatment were excluded from the study. Patients' clinical information is provided in detail in the following (Table 1).

Table 1. Clinical information for breast cancer samples.

Characteristic	Number (N=30)
Age	
<50	13
>50	17
Gender	
Male	0
Female	30
Grade	
I	9
II	9
III	12
Metastasis	
Positive	15
Negative	15

RT-qPCR

The samples were washed three times with phosphate-buffered saline (PBS) to remove contaminating and necrotic cells. RNA was extracted using TRIzol™ reagent (Invitrogen) according to the manufacturer's instructions. DNase I treatment (Sigma-Aldrich) was performed to remove DNA contamination. cDNA was synthesized using a cDNA synthesis kit (Takara) according to the manufacturer's instructions. Specific primers of *LncRNA WWTR1-AS1* (F: 5'-TATTTGGTCTGGCGAGGAGGC, R: 5'-TATTGCACTCAAGCCCGTTGG) were designed using Oligo7 software and confirmed using the Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). RT-qPCR with specific primers and SYBR Green was employed to measure the expression of LncRNA WWTR1-AS1 in breast cancer and adjacent normal tissues. Gene expression in each sample was calculated based on -ΔΔCt, and GAPDH was used as an internal control gene. RT-qPCR was programmed as follows: primary denaturation (95°C/15 min), followed by 40 cycles: denaturation (95°C/15 sec), annealing (60°C/20 sec), and extension (72°C/30 sec), and finally, Melt curve stage: 45 minutes Melt Curve (60 to 95 +0.3 °C).

Statistics and software

To compare gene expression in different groups, the normality of the data was checked using the Kolmogorov-Smirnov test. Considering that the expression of genes in different groups did not have a normal distribution, the Wilcoxon test was used to compare the gene expression of healthy and sick individuals. The Mann-Whitney test was used to compare the gene expression of people with and without metastasis, and the Kruskal-Wallis test was used to compare different degrees of the disease. Dunn's test was used as a follow-up test to compare the two groups after the Kruskal-Wallis test (Dinno, 2015).

A receiver operator characteristic (ROC) curve was used to show the ability of the studied gene to diagnose healthy and sick people. The area under the ROC curve provides an overall measure of a classifier's performance across all possible classification thresholds. All statistical analyses were performed using R statistical software, and version a false discovery rate (FDR) level of <0.05 was considered in all analyses. The network of expression and connection of genes with discovered lncRNAs was visualized using Cytoscape (V4) software, and the significance between groups was checked using a t-test.

Results

Deferentially expressed protein

For the identification of the differentially expressed protein in breast cancer versus normal tissue, a proteomics dataset including six primary tumors and seven non-tumoral samples was chosen for re-analysis using MaxQuant software. PCA and hierarchical clustering were performed to verify the sample grouping. The PCA plot showed that the non-tumor and tumor samples formed two separate clusters (Supplement 1A). Heretical clustering (heatmap) showed satisfying segregation of the quality of samples by utilizing FDR<0.05 as the statistical threshold. A total of 3408 proteins were identified by mass spectrometry. After filtration with a Q-value<0.05, 1149 DEPs remained, comprising 191 downregulated proteins and 958 upregulated proteins (Supplement 1B).

Top regulatory factors

To gain a better understanding of the regulatory factors in breast cancer, a multilayer complex network analysis was employed. The results revealed that 76 TFs, 61 kinases, 366 miRNAs, and 162 lncRNAs control 1149 DEPs. Based on the DEPs and their associated regulatory agents, a multi-regulatory network with 1811 nodes and 4022 edges, including DEPs, TFs, Kinases, and their regulatory miRNAs, and long noncoding RNAs, was constructed (Supplement 2). The top ten hub molecules in each layer were selected based on Cytoscape network analysis, as well as three topological parameters, including degree, betweenness, and closeness (Supplement 3).

According to the results WWTR1-AS1, hsa-miR-16-5p, Paraoxonase1 (PON1), mitogen-activated protein kinase 14 (MAPK14), and hepatocyte nuclear factor 4 alpha (HNF4A) are among the most effective factors in breast cancer.

Potential regulatory mechanisms

To detect the biological information related to quantified DEPs and predicted regulatory elements, a functional enrichment analysis was carried out in three sections, including Biological Process (BP), Molecular Function (MF), and Cellular Component (CC). GO analysis revealed that the most strongly enriched terms in biological processes (BPs) were negative regulation of gene expression, the metabolic process of mRNA, response to an organic substance, establishment of localization in the cell, protein-containing complex assembly, cellular ketone metabolic process, ribose phosphate metabolic process, and neutrophil activation. Based on the GO-MF results, it appears that DEPs are mainly implicated in cadherin binding, mRNA binding, magnesium ion binding, and protein serine/threonine kinase activity. In GO-CC, four significantly enriched terms included extracellular exosomes, proteasome complex, focal adhesion, and collagen-containing extracellular matrix, which are often associated with DEPs, TFs, and kinases (Supplement 4).

Altered pathways

Metascape analysis was performed to explore altered -biomedical phenomena related to DEPs, TFs, and kinases. The results showed that 11 pathways were mainly enriched, including the metabolism of RNA, neutrophil degranulation, VEGFA-VEGFR2 signaling pathway, diseases of signal transduction by growth factor receptors and second messengers, signaling by Rho GTPases, RNA Splicing, homeostasis, vesicle-mediated transport, protein processing in endoplasmic reticulum and processing of capped intron-containing pre-mRNA (Fig. 1).

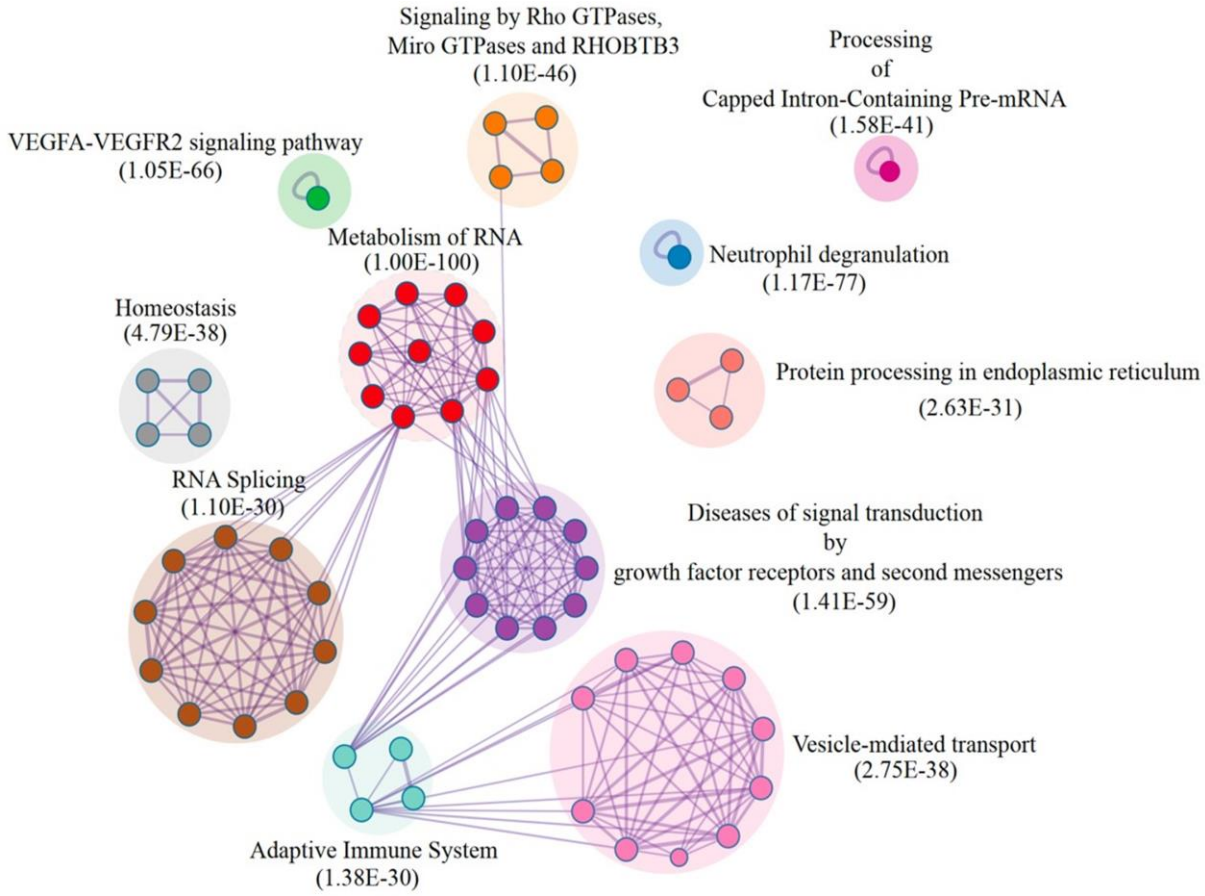

Fig. 1. Pathway enrichment analysis: Signalling pathway enrichment was executed based on DEPs, TFs, and kinases. All summarized enriched terms were selected based on FDR<0.05.

Functional enrichment analysis of lncRNA WWTR1-AS1 and its target genes

Our results introduced lncRNA WWTR1-AS1 as an effective lncRNA in the regulation of DEPs in breast cancer based on multilayer complex network analysis. GO and pathway enrichment analyses were performed to clarify and explore the functional role of WWTR1-AS1 in breast cancer. The results demonstrated that this lncRNA regulates some of the proteins and, in this way, plays a key role in focal adhesion, cell-substrate adhesion, cell-substrate junction assembly, organization of the actin cytoskeleton, morphogenesis of cellular components, and extracellular matrix (ECM)-associated interactions (Fig. 2).

LncRNA WWTR1-AS1 expression pattern and the ability for a reliable biomarker

To determine the expression pattern of WWTR1-AS1 lncRNA in breast cancer, the expression level of this lncRNA was evaluated in 30 paired tumor and normal samples.

The RT-qPCR results indicated that the expression of WWTR1-AS1 substantially increased in tumor samples compared to that in adjacent normal tissues (Fig. 3 A, B). RT-qPCR analyses showed that the lncRNA had an increasing trend in metastatic patients compared to non-metastatic patients (Fig. 3C). In addition, there is a positive correlation between increased expression of WWTR1-AS1 lncRNA and breast cancer grade (Fig. 3D). Because of the remarkable alterations in WWTR1-AS1 lncRNA expression, we performed ROC curve analysis to determine whether WWTR1-AS1 lncRNA is appropriate for testing the ability of the molecule to distinguish between normal and tumor status. Consequently, ROC analysis revealed that WWTR1-AS1 lncRNA could be a reliable biomarker to identify cancerous tissues in comparison to healthy ones, with (Area under the ROC Curve) AUC= 0.7750 and P= 0.0003 (Fig. 3 E).

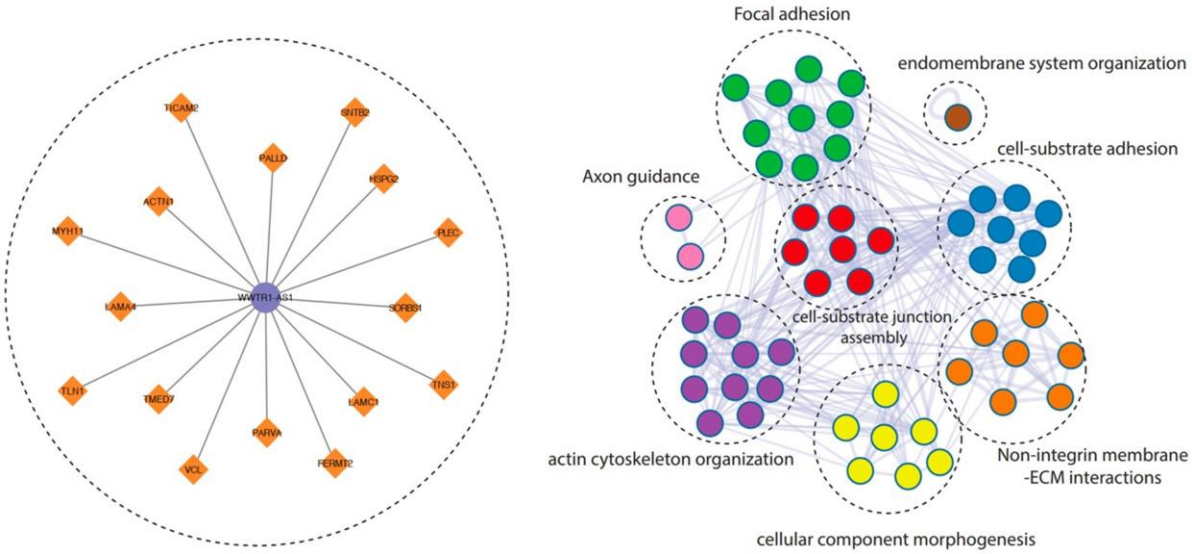

Fig. 2. Functional enrichment analysis: Functional enrichment analysis revealed that lncRNA WWTR1-AS1 target genes also detected regulatory mechanisms that are affected by lncRNAs' target genes.

Discussion

Despite the use of high-throughput approaches for the treatment of breast cancer, it remains one of the most lethal cancers worldwide (Siegel *et al.*, 2022). The increase in breast cancer rates may be due to the lack of screening methods for early detection of the disease. Additionally, the absence of comprehensive information about the responsible pathogenesis mechanisms is another factor that can be added to the list (Lehmann *et al.*, 2021). Therefore, a comprehensive characterization of the genetic and cellular alterations related to tumor genesis and progression is necessary for the development of novel cancer therapies and early detection. One of the malignant characteristics of cancer cells is the considerable alteration in protein expression levels. These proteins are involved in signaling and metabolic pathways, DNA repair, apoptosis, protein synthesis and degradation, and other cellular processes (Pessoa *et al.*, 2022). Identification of differentially expressed proteins in breast cancer is important for the discovery and validation of novel biomarkers, early diagnosis, breast cancer grading, measurement of disease progression, etc (Lu *et al.*, 2021; Neagu *et al.*, 2022). In this study, using systematic analysis, we found that among the differentially expressed proteins, more than 83% were increased in breast cancer and 17% were decreased.

Alterations in protein expression levels can be influenced by various factors, including transcription factors, kinases, miRNAs, and lncRNAs (Thomassen *et al.*, 2008; García-Aranda and Redondo, 2017; Loh *et al.*, 2019; Sideris *et al.*, 2022). A multilayer complex system can be used to investigate protein-

associated factors and identify molecules that can serve as cancer biomarkers (Zhang *et al.*, 2019b; Lu *et al.*, 2021). This system builds a comprehensive regulatory network and identifies central molecules. In addition, the multilayer systems have introduced potential drug targets and decoded the mechanisms of breast cancer pathogenesis. As part of our analysis, we identified DEP-related regulatory elements, including TFs, kinases, miRNAs, and lncRNAs. Functional enrichment analyses revealed that DEPs and their regulatory components were mainly involved in the negative regulation of biological processes, molecular functions, and cellular components.

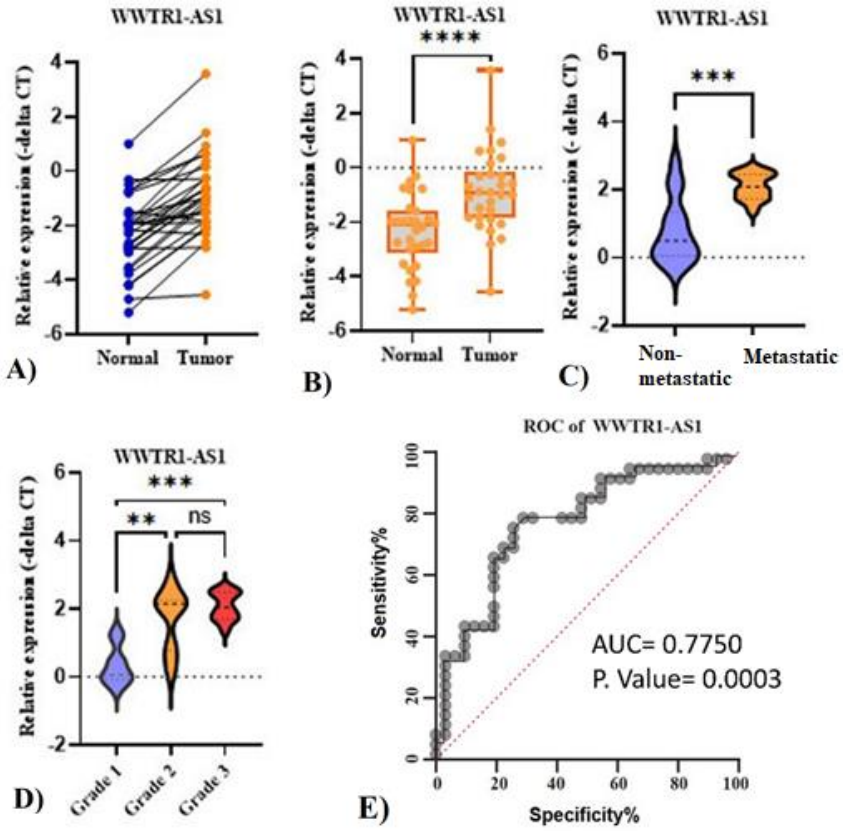

Fig. 3. Expression analysis of WWTR1-AS1: A and B) RT-qPCR result showed a relative expression level of WWWTR1-AS1 lncRNA in breast cancer tissues; C and D) The result of lncRNA expression level in metastasis patient compared with non-metastasis patient and in different breast tissue grades; E) ROC analysis result from WWTR1-AS1 lncRNA for evaluation its ability for a reliable biomarker in breast cancer.

Although genetic changes in the signaling pathways that regulate cell -cycle progression, apoptosis, and cell proliferation are typical characteristics of cancer, the degree, causes, and co-occurrence of changes in these pathways vary between individuals and tumor types (Sanchez-Vega *et al.*, 2018). Metascape analysis revealed pathways that are affected by candidate genes and other regulatory factors (Yu *et al.*, 2020). In the present study, we showed that DEPs, TFs, and kinases are involved in 11 pathways, six of which are related to each other, such as Signaling by Rho GTPases, RNA metabolism, and RNA splicing. Improvements in high-throughput sequencing methods have demonstrated that ncRNAs, especially lncRNAs and miRNAs, play an important role in tumorigenesis (Loh *et al.*, 2019; Sideris *et al.*, 2022; ZadehRashki *et al.*, 2022). LncRNAs regulate protein expression levels before and after transcription (Sideris *et al.*, 2022). The lncRNA WWTR1-AS1 is overexpressed in cervical squamous cell carcinoma (CSCC), and its upregulation increases Notch receptor 3 (Notch3) expression and stemness in CSCC, but its effect is reversed by miR-136 overexpression

(Zhou *et al.*, 2024). Likewise, abruption in the expression of WWTR1-AS1 and its significant correlation with the alteration of tumor size has been demonstrated. It has been observed that the increase in the expression of WWTR1-AS1 in head and neck cancer increases tumorigenesis in this cancer with malignant features and an unfavorable prognosis, while its inhibition suppresses head-neck squamous cell carcinoma proliferation, migration, invasion, and induced apoptosis (Li *et al.*, 2019a). In the present study, we selected the top 10 hub molecules in the multi-layer network based on the finding that WWTR1-AS1 had the highest expression between lncRNAs in tumor samples compared with normal samples.

In addition, functional enrichment analysis of WWTR1-AS1 lncRNA validated that its targets were mainly enriched in the regulation of focal and cell adhesion phenomena, underscoring the aforementioned findings. RT-qPCR results showed that WWTR1-AS1 lncRNA was upregulated in breast cancer tissues and increased in metastatic samples. In addition, lncRNA expression showed a significant increase in high-grade tumors compared to low-grade tumors. Following that, ROC analysis showed that WWTR1-AS1 could be used as a prognostic indicator.

Conclusion

Taken together, based on the bioinformatic analysis, we identified key DEPS, TFs, kinases, lncRNAs, and miRNAs that are involved in the underlying pathogenic mechanisms and regulatory pathways of breast cancer. We also introduced lncRNA WWTR1-AS1 as an effective factor in the regulation of DEPs and various regulatory mechanisms. Additionally, molecular evidence confirmed a high expression level of lncRNA WWTR1-AS1 in high-grade and metastasis breast tissues. Our results introduce lncRNA WWTR1-AS1 as a novel diagnostic biomarker for breast cancer for further investigation.

Ethics approval and consent to participate

All experiments were performed in accordance with relevant guidelines and regulations. Informed consent was obtained from all the donors prior to the collection of samples.

Disclosure Statement

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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