

Expression Analysis and Bioinformatics-Based Identification of miR-4534, miR-551a and miR-1294 in Breast Cancer

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ABSTRACT This study aimed to ascertain the potential role of specific microRNAs (miR-551a, miR-1238, miR-4534, and miR-1294) in breast cancer by analysing their expression levels and identifying the target genes and pathways they regulate. Expression levels in 82 samples from 41 patients were measured using quantitative Real-Time PCR. Bioinformatics tools were utilised to identify the potential target genes and pathways. Findings revealed that miR-1294 and miR-551a had significantly lower expression in tumour tissues compared to normal tissues, while miR-4534 was overexpressed in tumour tissues. No significant difference was observed in miR-1238 expression between the two. miR-1294, miR-551a, and miR-4534 were found to regulate genes associated with Mapk, ErbB, Hippo Wnt, EGFR, and P53 signalling pathways. The study concludes that miR-551a, miR-1294, and miR-4534 may play a role in the development of breast cancer.

INTRODUCTION

Breast cancer (BC) is a leading invasive malignancy among women and has garnered considerable global attention (Barzaman et al. 2020). Breast cancer is a highly frequent cause of prevailing deaths in women worldwide. According to the World Health Organization (WHO) statistics report of 2020, breast cancer was found in 2.3 million and 685,000 deaths in 2020. By 2040, due to population growth and aging, annual cases are expected to exceed 3 million with 1 million deaths (Arnold et al. 2022). Notwithstanding the strides in early detection and therapeutic interventions, approximately one-third of breast cancer patients grapple with disease progression and metastasis during treatment (Leone and Leone 2015). The heterogeneity of breast cancer tumours poses formidable challenges to therapeutic innovation. While existing chemotherapeutic regimens have improved survival rates, there is a propensity for some breast tumours to develop resistance post-initial response. Consequently, it is imperative to elucidate molecular targets within breast cancer that may be pivotal for the genetic modulation of the disease and sensitise tumours to therapeutic agents (Masuda et al. 2012; Bailleux et al. 2021).

MicroRNAs (miRNAs) are a subset of non-coding RNAs (ncRNAs) consisting of 19 to 24 nucleotides, and changes in their expression levels have been implicated in the pathogenesis and metastasis of various cancer types. Accumulating evidence has unveiled the capacity of miRNAs to induce anomalies in gene expression, culminating in the initiation and progression of cancer (Reddy 2015). These miRNAs are known to modulate key signalling pathways either by upregulation or downregulation, engendering alterations in the expression profiles of critical genes and hence fostering tumorigenesis (Tao et al. 2021). Cellular homeostasis, survival, and apoptosis are orchestrated by myriad signalling pathways that underpin a cascade of biological events and gene expression. Though these signalling pathways do not engage in direct transcriptional regulation of many genes, they indirectly influence gene expression profiles, thus subverting cellular functions (Jonathan et al. 2017; Xu et al. 2020). Research endeavours have shed light on the role of miRNAs in these pathways, with several investigations unravelling correlations between miRNA dysregulation and breast cancer phenotypes regarding cancer initiation, progression, and metastasis (Petri and Klinge 2020).

In the literature, miR-1294 has been implicated in the abnormal expression profiles in oral squamous cell carcinoma, glioma, gastric cancer, ovarian cancer, and osteosarcoma by targeting genes such as insulin-like growth factor 1 receptor (IGF1R), MYC proto-oncogene (c-MYC), homeobox A6 (HOXA6) (Wang et al. 2018; Zhang et al. 2018; Pan et al. 2019; Wang et al. 2020; Mao et al. 2023). The downregulation of miR-1294 expression has been observed to stimulate proliferation, hinder apoptosis, and augment the invasiveness and migration of cancer cells. It also appears to be involved in the activation of crucial cancer-related signalling pathways such as Wntless-related integration site (Wnt), phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) and rat sarcoma (RAS), thereby promoting cancer development. Notably, an association has been found between the downregulation of miR-1294 and a worse prognosis in oesophageal squamous cell carcinoma, gastric cancer, epithelial ovarian cancer, pancreatic ductal adenocarcinoma, breast, and non-small cell lung cancer (Li et al. 2023; Mao et al. 2023).

Similarly, miR-551a has exhibited tumour-suppressive activities across various cancer types, such as colorectal cancer, lung cancer, gastric cancer, and osteosarcoma, acting as a potential tumour suppressor by targeting phosphatase of regenerating liver 3 (PRL-3) and creatine kinase B (CKB) genes (Loo et al. 2015; Du and Sha 2017). Another study demonstrates that miR-551a was downregulated in breast cancer cells by targeting the FBJ murine osteosarcoma viral oncogene homolog (c-fos) gene (Anuj et al. 2019). In ovarian cancer, miR-551a was reported to inactivate the phosphoinositide 3-kinases/serine/threonine kinase (PI3K/Akt) signalling pathway by downregulating insulin receptor substrate 2 (IRS2) (Du and Sha 2017). Also, targeting miR-551a could have implications for the resistance of cancer cells to anti-cancer drugs like 5-Fluorouracil (5-FU) (Kang et al. 2019).

Contrastingly, miR-4534 has been the subject of numerous studies examining its overexpression in various types of cancer. Notably, a study based on the Gene Expression Omnibus database reported an upregulation of miR-4534 in breast cancer, targeting ADAM-like Decysin-1 (ADAMDEC1) (Chai et al. 2023). This upregu-

lation has been associated with poor overall survival in breast cancer patients, pointing towards the critical role of miR-4534 in tumour progression. Mir-4534 is also overexpressed in prostate cancer, profoundly impacting cell proliferation, migration, inducing G0/G1 cell cycle arrest and apoptosis. This overexpression affects tumour tissue growth and could provide a promising therapeutic target for the treatment of PCA in the future (Jiang et al. 2020). Furthermore, miR-4534 has been found to block the Phosphatase and Tensin homolog (PTEN) pathway, known for its interaction with autophagy-related 2B (ATG2B) genes, contributing to the complexity of prostate and colorectal cancers. This upregulation and interaction highlight the potential role of miR-4534 in promoting prostate cancer cell proliferation, migration, invasion, and angiogenesis by inhibiting vasohibin 1 (VASH1) expression (Nip et al. 2016; Inoue et al. 2021). miR-1238 has manifested abnormal expression in non-small cell lung cancer through its targeting of LIM Homeobox 2 (LHX2) with other investigations documenting distinct regulatory patterns for miR-1238 in cervical and prostate cancers (Shi et al. 2015; Shan et al. 2020; Li et al. 2021).

These studies underscore the involvement of miR-1294, miR-551a, miR-1238, and miR-4534 in various cancer types. However, these miRNAs' exact cellular roles and molecular targets remain definitively ascertained. A more comprehensive understanding of these miRNAs necessitates further investigation.

Objective

This study aims to explore the roles of miR-1294, miR-551a, miR-1238, and miR-4534 in the context of breast cancer, extending beyond their established involvement in various cancer types. This study seeks to provide a better understanding of these miRNAs' specific cellular functions, molecular targets, and mechanistic dynamics within the broader genomic landscape of breast cancer. Moreover, it aspires to uncover the potential relationships between these miRNAs with key cancer-related signalling pathways and to evaluate the implications on therapeutic resistance. This research strives to fill a critical knowledge gap and contribute to the broader understanding of breast cancer pathogenesis, potentially leading

to the identification of novel therapeutic approaches and prognostic biomarkers.

MATERIAL AND METHODS

Tissue Samples

The study encompassed an analysis of 82 tumour and histologically adjacent normal tissue samples (distance from the tumour greater than or equal to 5 cm), which were procured from 41 female breast cancer patients who visited Gaziantep University Sahinbey Research and Practice Hospital, Department of General Surgery between 2020 and 2021. These patients were not undergoing chemotherapy or radiotherapy at the time of sampling. A written, signed, and informed consent was obtained from all participants before tissue sampling. The tissue samples were preserved in tubes containing RNA Later solution and stored at -80 °C until further analysis. Gaziantep University Local Ethics Committee provided approval for this study. In this study¹, as mentioned in (Table 1), 21 patients (51%) were 50 years or older, and 20 (49%) were younger than 50 years. The majority were in the early stages of cancer, with 28 patients (68%) categorised in stages I and II, while 13 patients (32%) were in stages III and IV. Only two patients (5%) reported smoking, and none consumed alcohol. Tumour localisation was evenly distributed, with 21 patients (51%) having tumours on the right side and 20 patients (49%) on the left. Most patients (38 or 93%) had no distant metastasis (M0), while only 3 patients (7%) had metastatic cancer (M1). In terms of tumour size, most patients (34 or 83%), had tumours less than 50 mm, while seven patients (17%) had tumours of 50 mm and above. Among these patients, 4 (10%) reported a family history of cancer, while 37 (90%) did not. For the hormone receptors status, 30 patients (73%) showed positive estrogen receptor (ER), and 11 patients (27%) were negative. For progesterone receptors (PR), 29 patients (71%) were positive, and 12 patients (29%) were negative. Regarding the histological types of breast cancer, the majority, 33 patients (80%), had invasive ductal carcinoma, four patients (10%) had invasive lobular carcinoma, three patients (7%) had invasive breast carcinoma, and 1 patient (2%) had invasive med-

Table 1: Characteristic features of the breast cancer patients analysed in this study.

<i>Clinicopathological factor</i>	<i>Number</i>	<i>Percentage</i>
<i>Phase</i>		
I and II	28	68
III and IV	13	32
<i>Age</i>		
≥ 0 years	21	51
< 50 years	20	49
<i>Gender</i>		
Man	0	0
Woman	41	100
<i>Smoking</i>		
Yes	02	5
No	39	95
<i>Alcohol use</i>		
Yes	0	0
No	41	100
<i>Tumour Localisation</i>		
Right	20	51
Left	21	49
<i>Distant Metastasis</i>		
M0 (No Metastasis)	38	93
M1 (Metastasis)	03	7
<i>Family History</i>		
Yes	04	10
No	37	90
<i>Tumour Size (mm)</i>		
Less than 50mm	34	83
50mm and above	7	17
<i>Lymph Node Metastasis</i>		
N1	33	80
N2	5	12
N3	3	7
<i>ER (Estrogen receptor)</i>		
Positive	30	73
Negative	11	27
<i>PR (Progesterone receptor)</i>		
Positive	29	71
Negative	12	29
<i>CEA</i>		
Normal	34	83
High	7	17
<i>CA-125</i>		
Normal	38	93
High	03	7
<i>CA 15-3</i>		
Normal	40	98
High	1	2
<i>C-erbB-2</i>		
+1	4	10
+2	6	15

ullary carcinoma. For lymph node metastasis, 33 patients (80%) were in stage N1², 5 patients (12%) in N2³, and 3 patients (7%) in N3⁴. In terms of cancer markers, 34 patients (83%) had normal levels of carcinoembryonic antigen (CEA⁵), while 7 patients (17%) had abnormal levels. Cancer

Antigen 125 (CA-125⁶) levels were normal in 38 patients (93%) and high in 3 patients (7%). Cancer antigen 15-3 (CA15-3⁷) levels were normal for 40 patients (98%), with only 1 patient (2%) showing high levels. For receptor tyrosine-protein kinase (C-erbB-2), 4 patients (10%) were scored at +1, 6 patients (15%) at +2, 11 patients (27%) at +3, and 20 patients (49%) were negative. Regarding the Ki-67 index⁸, 1 patient (3%) was in grade G1⁹, 19 patients (46%) in G2¹⁰, and 21 patients (51%) in G3¹¹.

MicroRNA Extraction and Quantification

Total RNA from fresh frozen tissue samples was extracted using the Hybrid-R™ miRNA isolation kit (GeneAll, South Korea) per the manufacturer's instructions. The RNA was quantified using a MaestroNano Spectrophotometer (Maestro-gen), and the RNA concentrations were adjusted to a standard concentration for uniformity across samples. The samples were then stored at -80°C until further use.

Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Total RNA was reverse transcribed into complementary DNA (cDNA) using the Taq-Man™ Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific, USA) following the manufacturer's protocol. The synthesised cDNA samples were stored at -80°C until needed. The 7500 Fast Real-Time PCR system (Applied Biosystems) was used for qPCR with a Single miRNA qPCR Assay kit (Thermo Fisher Scientific, USA). The relative expression levels of miR-551a, miR-1238, miR-1294, and miR-4534 were normalised against RNU6NB as the control gene. Each qPCR analysis was conducted in triplicate, and relative expression levels were calculated using the $\Delta\Delta C_t$ method, which calculates fold change in gene expression levels by normalising against a housekeeping gene and comparing it to a control sample.

Statistical Analysis

The evaluation of the association between clinical and pathological data (categorical variables) and miRNA expression levels was performed using Pearson's Chi-Square and Fisher's Exact tests. Statistical analysis was conducted using SPSS software (version 22.0). Additionally, a paired t-test was used to compare the

expression levels of miRNAs between tumour tissues and their normal counterparts. A p-value of less than 0.05 was considered significant. The relative gene expression, indicated as fold change, was determined using the $\Delta\Delta C_t$ method.

microRNA-mRNA Target Identification

The bioinformatics analysis was conducted only on miRNAs that exhibited significant differences in expression analysis between the tumour and the normal tissues. Targets of the microRNAs were identified using two online databases, miRWalk (Sticht et al. 2018) and miRDB (Chen and Wang 2020). A target score threshold of greater than 80 was used, as suggested by miRDB. Targets scored higher than 0.95 and listed in at least two databases were selected. A combined list of targets from miRDB and miRWalk was created and used for further downstream analysis.

Functional Enrichment Analysis of microRNAs

Functional enrichment analysis, an essential step in transcriptomic studies, was conducted to assess the enrichment of genes in various biological functions and processes (Webber 2011). The EnrichR R package used the generated target list as input for this analysis. Parameters for enrichment were set to include libraries such as Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and PANTHER (Thomas et al. 2022).

Protein-Protein Network Analysis

Protein-protein interaction networks (PPINs) were analysed using the STRING database, which provides information on known and predicted protein-protein interactions (Nadaradjane et al. 2018).

RESULTS

Expression of miR-551a, miR-1294, miR-4534, and miR-1238 and their Correlation with the Clinicopathological Factors

The average ΔC_t value of miR-551a was noted to be -1.36 ± 0.38 for tumour tissues and -1.7 ± 0.9 for normal tissues, with a corresponding fold change of 0.84 ± 0.38 . Notably, miR-551a expression was significantly downregulated in tumour tissues compared with the normal tissues

($p=0.038$) (Fig. 1a). In contrast, the average ΔCt value of miR-1294 was observed to be 0.4 ± 0.24 in tumour tissues and 0.1 ± 0.58 in normal tissues, resulting in a fold change of 0.82 ± 0.13 . A statistically significant reduction in the miR-1294 expression level in tumour tissues relative to normal tissues was evident ($p=0.004$) (Fig. 1b). This implies that the tumour microenvironment could potentially suppress miR-1294 and miR-551a, culminating in diminished expression in tumour samples compared to normal samples. Regarding miR-4534, the average ΔCt value was -5.39 ± 0.43 in tumour tissues and -4.86 ± 1.02 in normal tissues, with a fold change of 1.5 ± 0.43 . A statistically significant elevation in the expression level of miR-4534 was documented in tumour tissues relative to normal tissues ($p=0.003$) (Fig. 1c), thus, suggesting an upregulation in the tumour samples. For miR-1238, the average ΔCt value was registered as 2.29 ± 0.75 in tumour tissues and 2.52 ± 0.71 in normal tissues, with a calculated fold change of 1.35 ± 0.78 . There was no statistically significant difference in the miR-1238 expression level between tumour and normal tissues ($p=0.076$) (Fig. 1d). Insights regard-

ing miR-1294, miR-4534, and miR-1238 are pioneering, as these miRNAs have not been previously studied in the context of breast cancer, thereby suggesting their potential as novel biomarkers for this malignancy. A chi-square test was conducted to examine the potential correlation between the expression levels of miR-551a, miR-1294, miR-1238, and miR-4534 and various clinicopathological factors among breast cancer patients. The factors considered in the analysis included age, smoking, alcohol use, family history, phase, histological type, tumour size, tumour localisation, distant metastasis, lymph node metastasis, and other breast cancer biomarkers such as ER, PR, CEA, CA-125, CA 15-3, C-erbB-2, and Ki-67.

The analysis results indicated no statistically significant correlation between the clinicopathological factors and the expression levels of miR-551a, miR-1294, miR-1238, and miR-4534 ($p > 0.05$). These findings suggest that the examined clinicopathological factors may not substantially influence the expression levels of these miRNAs in the sample of breast cancer patients studied (Appendix I).

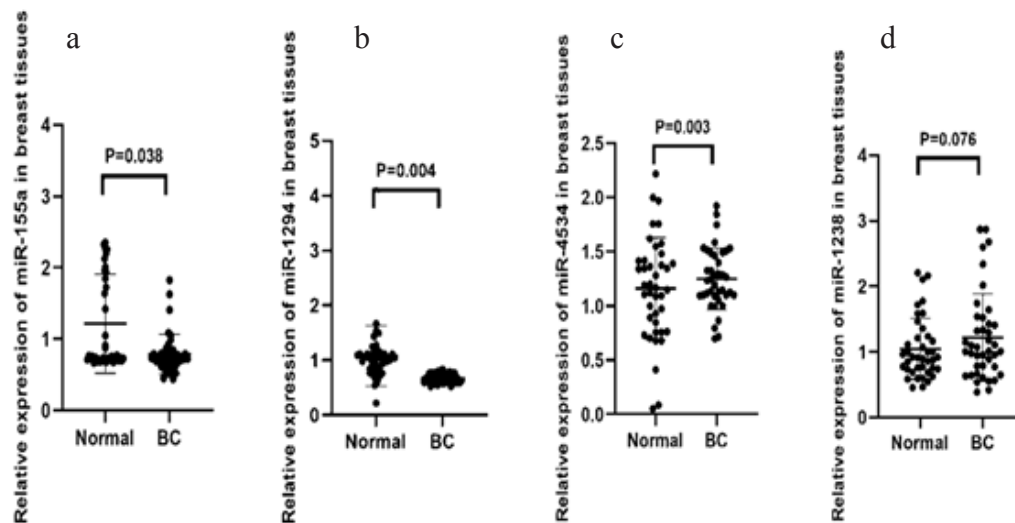


Fig. 1. Relative expression of miRNAs in breast tissues compared to normal ones using qRT-PCR, all statistical analysis was performed using a student t-test on SPSS, * $p < 0.05$, ** $p < 0.01$. (a) miR-155a was downregulated in breast cancer tissues $p < 0.05$; (b) miR-1294 was downregulated in breast cancer tissues $p < 0.05$; (c) miR-4534 was overexpressed in breast tissues $p < 0.05$; (d) miR-1238 expression was not statically significant $p > 0.05$

MicroRNA-mRNA Target Identification

This study identified target genes associated with the downregulated miRNAs, that is, miR-1294 and miR-551a, as well as the upregulated miRNA of miR-4534. The selection criteria involved utilising a target score exceeding 80 in the miRDB database and ensuring the presence of genes in both TargetScan and miRDB databases, verified through the miRWalk database. Subsequently, unique sets of target genes were synthesised by integrating the gene lists from the respective databases for further analysis. For miR-1294, an initial pool of 163 target genes was extracted from miRDB. Upon cross-referencing with miRWalk, 44 genes were found to be shared. This aggregation led to a culminating set of 168 target genes, inclusive of BRCA1/BRCA2-containing complex subunit 3 (BRCC3), MYC, MYCL, cyclin D2 (CCND2), and deubiquitinase YOD1 (YOD1). In the case of miR-551a, miRDB contributed 5 target genes, with miRWalk revealing 56 that were in common with TargetScan and miRDB databases. This resulted in a definitive collection of 18 target genes, with notable examples being Crumbs Cell crumbs 2 (CRB2), myocyte-specific enhancer factor 2C (MEF2C), Erb-b2 receptor tyrosine kinase 4 (ERBB4), transmembrane protein 2 (TREM2), and transmembrane protease, serine 2 (TMPRSS2). With respect to miR-4534, 168 target genes were identified in the miRDB database. Cross-validation through miRWalk indicated 124 genes in common, culminating in an assemblage of 193 target genes, SRY-box transcription factor 6 (SOX6), adenylate cyclase 1 (ADCY1), dishevelled associated activator of morphogenesis 2 (DAAM2), oestrogen-related receptor gamma (ESRRG) and forkhead box Q1 (FOXQ1), zinc finger protein 142 (ZNF142). Supplementary materials (Bioinformatics analysis)

Gene Ontology and Pathway Analysis

In the pursuit of comprehending the underlying mechanisms of breast cancer, this investigation elucidated the implications of miR-1294, miR-551a, and miR-4534 through rigorous GO and KEGG pathway analyses. The presented results consider statistically significant findings, with p-value less than 0.05.

The study on miR-1294 indicated an array of genes associated with distinct cellular components such as RNA-induced silencing complex (RISC) complex and collagen type IV trimer. The molecular function analysis revealed that protein tyrosine phosphatase activity and transcription corepressor binding were significantly enriched in the genes regulated by miR-1294. Furthermore, KEGG pathway analysis indicated an aberration in multiple biological pathways, including Hippo signalling pathway and pathways in cancer, due to the downregulation of miR-1294.

Exploring the roles of miR-551a revealed enrichment in biological processes such as positive regulation of cardiac muscle cell proliferation and tissue growth. Target genes of miR-551a showed significant enrichment in aspartic-type endopeptidase inhibitor activity, cardiac muscle regulation, cell differentiation, circulatory system development, and minor groove of adenine-thymine-rich DNA binding, and hyaluronan glucosaminidase activity. KEGG pathway analysis indicated that the downregulation of miR-551a was correlated with the upregulation of genes involved in the mitogen-activated protein kinase (Mapk) signalling pathway, Notch signalling pathway, and erythroblastic leukemia viral oncogene homolog (ErbB) signalling pathway, and Sphingolipid metabolism pathway, suggesting a significant influence on these pathways.

The GO results of miR-4534 revealed its enrichment in diverse biological processes, including neuromuscular synaptic transmission and the biosynthetic process of chondroitin sulphate. Functions such as voltage-gated sodium channel activity, protein complex oligomerization, and sialyltransferase activity were significantly prevalent among genes regulated by miR-4534. KEGG pathway analysis indicated that miR-4534 potentially impacts multiple crucial biological pathways, including Glutamatergic synapses, Wnt, relaxin, and the PI3K-Akt signalling pathways (Appendix II).

Protein-Protein Interaction (PPI) Analysis

Investigating the role of miRNAs in breast cancer progression remains an integral aspect of research. This study delves into the interaction between miRNAs and their target genes through PPI analysis, revealing insightful findings.

Exploring miR-1294, a downregulated miRNA, unveiled top target genes BRCC3, CCND2, MYC, MYCL, and YOD1, were implicated in critical processes like negative regulation of monocyte differentiation, histone deubiquitination, lys63-specific deubiquitinase activity and cyclin binding. The top targeted protein CCND2 of miR-1294 has been identified with many different genes, including Cyclin-dependent kinase 6 (CDK6), S-phase kinase-associated protein 2 (SKP2), Cyclin-dependent kinase 2 (CDK2), and Cyclin-dependent kinase 4 (CDK4), Figure 2. Further, the analysis indicated a significant association of these genes with the p53 signalling pathway.

The top target genes of the downregulated miR-551a, namely, ERBB4, MEF2C, CRB2, and TMRSS2, were identified to have a network with the most adverse functions related to the negative regulation of Interleukin-15 (IL-15)-mediated signalling pathway, ERBB signalling pathway, epidermal growth factor receptor (EGFR) binding, along with others. The top targeted protein, ERBB4, of miR-551a has been identified

with many different genes, including discs large homolog 4 (DLG4), growth factor receptor-bound protein 2 (GRB2), heparin-binding EGF-like growth factor (HBEGF), neuregulins (NRG) gene family, and SCH1, among others, in Figure 3. Most of the interactions of ERBB4 are with the Immunoglobulin (Ig)-like domain in the NRGs family of proteins.

The top target genes of the upregulated miR-4534, namely SOX6, ADCY1, DAAM2, ESRRG, FOXQ1, and ZNF142, were determined to have a network with a majority of functions related to the canonical Wnt signalling pathway, cAMP biosynthetic process, GTPase activity, G-protein beta/gamma-subunit complex binding, and regulation of lipolysis, among others. ZNF142 and FOXQ1 were found to have no interaction in the network, neither with each other nor with other proteins (Fig. 4). SOX6 was identified to have interactions with catenin beta 1 (CTNNB1), which further had interactions with other proteins such as dishevelled segment polarity protein 1 (DVL1), dishevelled associated activator of morphogenesis 2 (DAAM2), and dishevelled

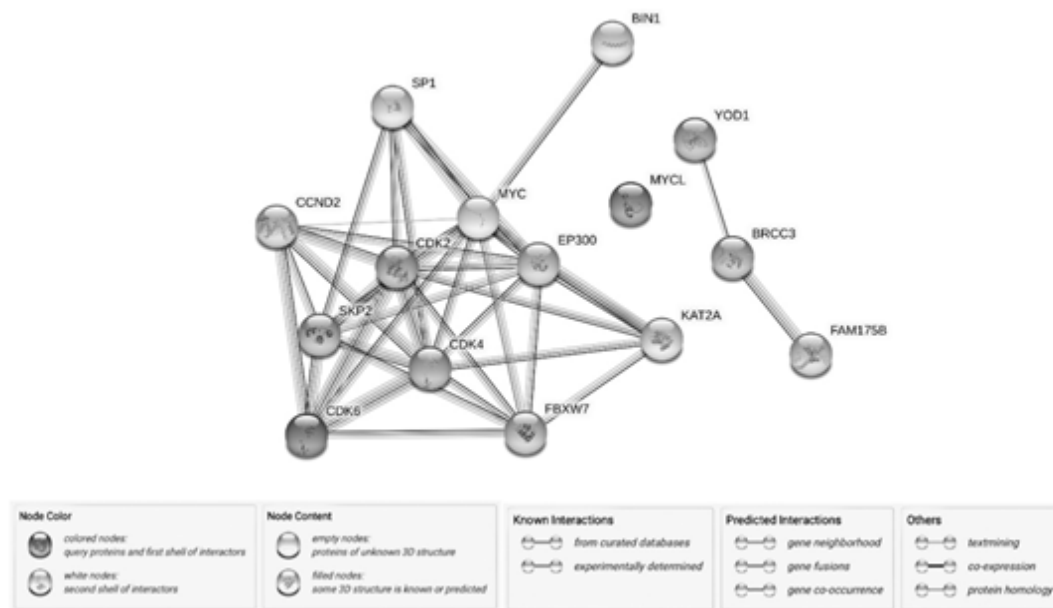


Fig 2. Protein-Protein Interaction analysis of miR-1294. Balls represent the proteins, whereas the edges represent the connections between the proteins. Here, CCND2 has many contacts with various other proteins.

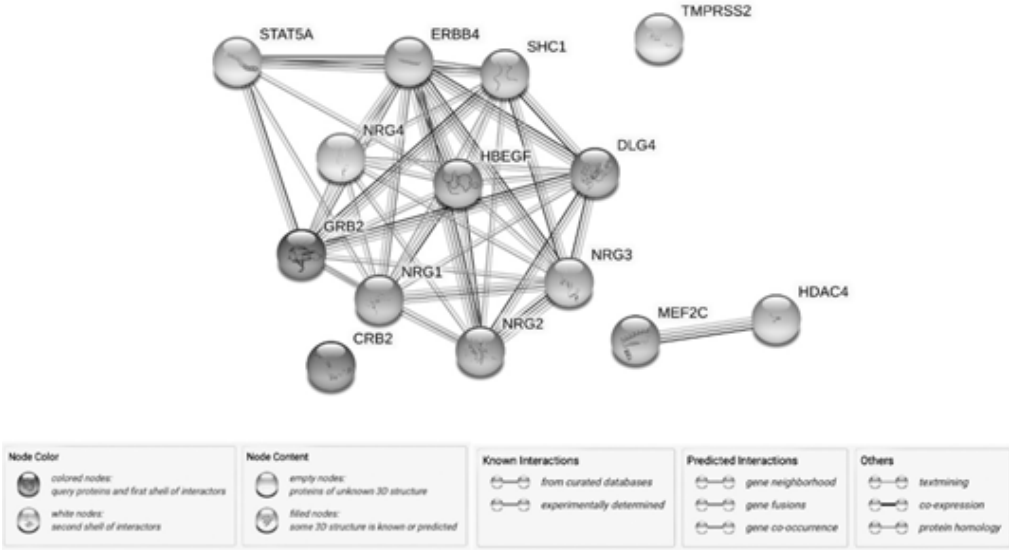


Fig. 3. Protein-Protein Interaction analysis of miR-551a. Balls represent the proteins, whereas the edges represent the connections between the proteins. Here, ERbB4 has many contacts with various other proteins, especially the NRG family

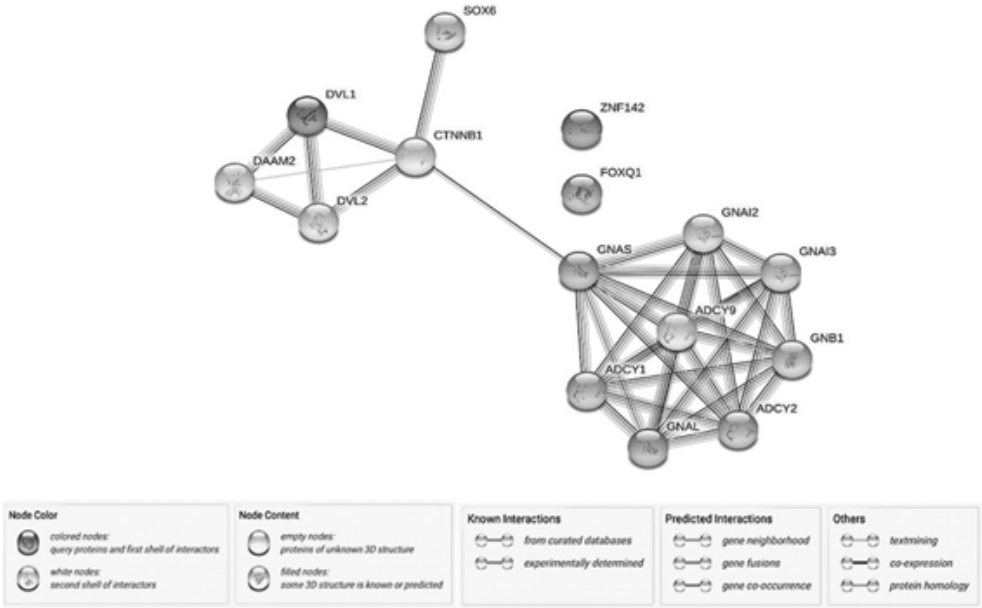


Fig. 4. Protein-protein Interaction analysis of miR-4534. Balls represent the proteins, whereas the edges represent the connections between the proteins. ADCY1 has many connections with various other ADCY protein family and G proteins family

segment polarity protein 2 (DVL2). On the other hand, adenylate cyclase 1 (ADCY1) was found to have interactions with the adenylate cyclase family of proteins, along with the G protein family, including G protein subunit alpha I2 (GNAI2) and others.

DISCUSSION

There are limited studies on the effect of miR-1294, miR-551a and miR-4534, in breast cancer tissues. By combining an asymmetrical pipeline of experimental technologies *in vitro* and *in vivo*, the researchers explored the role of these miRNAs in breast cancer progression and metastasis. Additionally, bioinformatics analysis was employed to study the dysregulated miRNAs in breast cancer tumours to determine their target genes, protein-protein interactions, and how these interactions berate the biological pathways and contribute to the proliferation and metastasis of cancer cells.

The current study prominently reveals the significant downregulation of miR-1294 in breast tumour tissues compared to normal tissues. This observation aligns well with previous reports demonstrating consistent downregulation of miR-1294 across a spectrum of 15 distinct malignancies, including oesophageal squamous cell carcinoma, gastric cancer, epithelial ovarian cancer, pancreatic ductal adenocarcinoma, and non-small cell lung cancer. The reported pathways for miR-1294 were PI3K/AKT/mTOR, RAS, and JAK/STAT pathways. The target genes of miR-1294 identified in this context are crucial in modulating vital signalling cascades like PI3K/AKT/mTOR, RAS, and JAK/STAT pathways. Furthermore, reduced miR-1294 expression associates with increased resistance to cisplatin and temozolomide (Mao et al. 2023).

Similar to the researchers' findings, a study reported downregulation of miR-1294 in breast cancer, inhibiting cellular proliferation, migration, and invasion by modulating the extracellular signal-regulated kinase (ERK) signalling pathway (Chen et al. 2022). In line with these observations, research has underscored the downregulation of miR-1294 in hepatocellular carcinoma relative to healthy tissues. The reduced expression level of miR-1294 facilitates the progression of hepatocellular cancer by attenuating the

inhibitory effects on its oncogenic targets, such as transforming growth factor beta receptor 1 (TGFBRI). As such, miR-1294 epitomizes a tumour suppressive role in hepatocellular carcinoma (Qin and Wang 2023). Notably, an anomaly was observed in a study of 15 children with acute lymphoblastic leukaemia, indicating an unexpected miR-1294 upregulation targeting SOX15 (Cen et al. 2023). Due to the small sample size used in this study, the reliability of the results is questionable.

These studies provide evidence for the tumour-suppressive properties of miR-1294 in various cancer types. To comprehend the mechanistic role of miR-1294 in breast cancer, the researchers conducted bioinformatics analyses which identified BRCC3, CCND2, MYC, MYCL, and YOD1 as target genes for miR-1294. BRCC3, a subunit of the BRCA1-BRCA2 complex involved in the DNA damage response, is upregulated in tumour tissues, and associated with cancer metastasis (Zhang and Zhou 2018). The overexpression of CCND2, MYC, and MYCL has been reported in breast cancer and other cancer types and shown to contribute to cancer progression (Xu et al. 2010; Flem-Karsen et al. 2018; Li et al. 2019). YOD1, although not reported in breast cancer, is overexpressed in other cancer types (Zhang et al. 2022). MYC was previously reported as a target for miR-1294 (Wang et al. 2018). The research findings reveal that in breast cancer, miR-1294 is downregulated, leading to the upregulation of BRCC3, CCND2, MYC, MYCL, and YOD1 genes. The GO and functional analysis of miR-1294's suggested target genes reveals its involvement in regulating lipid synthesis, cholesterol storage, Wnt signalling, GTPase activity, metalloproteinase activity, and cyclin binding. These processes have been linked to cancer development in various studies (Sukocheva and Wadham 2014; Litan and Langhans 2015; Fields and Stawikowski 2016; Sevinsky et al. 2018; Tauro and Lynch 2018; Wang et al. 2020). Furthermore, the conducted KEGG pathway analysis reinforces the roles of these genes in pathways such as the Hippo signalling pathway, mTOR signalling pathway, pathways in cancer, and Wnt signalling pathway. The involvement of miR-1294 in the Wnt signalling pathway and mTOR signalling pathway has been previously reported (Li et al. 2023; Mao et al. 2023). The Hippo pathway's influence on breast cancer,

essential for colonization, molecular biology, and treatment response, is underscored (Kyriazoglou et al. 2021). Dysregulation of Hippo signalling fosters breast cancer progression by interacting with the P53 oncogenic pathway, amplifying tumorigenesis potential (Raj and Bam 2019). The PPI analysis revealed that genes in the network are enriched in the p53 signalling pathway, vital in many cancers, including breast cancer. Aberrations in this pathway can drive the onset, growth, and spread of breast cancer (Duffy et al. 2018). The protein CCND2, targeted by miR-1294 and involved in the Hippo signalling pathway, interacts with several genes such as CDK6, SKP2, CDK2, and CDK4. Many of CCND2's interactions involve the cullins family, which serves as scaffolds for ubiquitin ligases (E3). These E3 ligases, vital for tumour suppressors, play a significant role in tumorigenesis. Furthermore, E3 has been associated with breast cancer cell invasion and migration through the p53 pathway (Ohta and Fukuda 2004; Wang et al. 2021). This study indicates that the downregulation of miR-1294 affects crucial biological pathways and functions as a tumour suppressor in breast cancer. Furthermore, the research highlights BRCC3, MYCL, CCND2, and YOD1 as potential targets of miR-1294, demonstrating the involvement of the P53 pathway in miR-129 regulation. Notably, the study reveals the previously unrecognized targeting of the Hippo signalling pathway and its associated gene CCND2 by miR-1294.

It is important to acknowledge the limitations of this study, notably the analysis of clinicopathological factors. Given the limited sample size, the researchers cannot confidently make associations between clinicopathological factors and miR-1294 expression and the other studied miRNAs. It should be emphasized that further research, particularly using *in vivo* experiments, is essential to determine the target genes and pathways associated with miR-1294. This is also pertinent to other miRNAs that are the focus of this study.

The expression analysis of the miR-551a revealed a significant downregulation of its expression in breast cancer tissues compared to adjacent normal tissues. This finding aligns with a previous study in ovarian cancer, which showed that miR-551a was downregulated and acted as a tumour suppressor by inhibiting cell prolifera-

tion, migration, and invasion. The target gene identified was IRS2, which is involved in the PI3K/AKT signalling pathway (Du and Sha 2017). Similarly, in breast cancer, miR-551a was found to be downregulated by upregulating the Focal adhesion kinase (FAK) pathway, an emerging therapeutic target in breast carcinoma (Anuj et al. 2019). In agreement with the current study, research on breast cancer reported miR-551a downregulation in breast cancer (Kang et al. 2019). Contrary to these findings, a study on miR-551a in patients with metastatic head and neck squamous cell carcinoma (HNSCC) presented divergent outcomes. The research indicated that miR-551a is upregulated and acts as an oncogene in this context, promoting processes such as cell proliferation, migration, invasion, and radio-resistance. The authors identified GLI pathogenesis-related 2 (GLIPR2) as a target gene of miR-551a in the context of HNSCC (Karanam et al. 2023). Another contrasting study demonstrated that miR-551a is upregulated in hepatocellular cancer, acting as a carcinogen, and correlating with poor overall survival (Chen et al. 2021). The complex nature of miR-551a's function, including oncogenic and tumour-suppressive properties, can be attributed to its diverse behaviours in different cancer types. These behaviours are determined by the tissue-specific context inherent to each malignancy. Bioinformatics analyses were conducted to comprehensively understand the molecular mechanisms associated with miR-551a in breast cancer. This study identified ERBB4, MEF2C, CRB2, and TMPRSS2 as the top genes potentially targeted by miR-551a. ERBB4, which encodes the epidermal growth factor (EGFR) receptor family member, has been associated with proliferative breast tumours and other cancer types (Hu et al. 2021; Kawahara and Simizu 2022; Schubert et al. 2023). CRB2, a crumbs cell polarity complex component that regulates embryonic development, is upregulated in glioblastoma, and may function as a carcinogen (Wang et al. 2022). TMPRSS2, a transmembrane serine protease, is overexpressed in breast cancer cells, potentially promoting carcinogenesis. Furthermore, TMPRSS2 expression is associated with poor prognosis in ER-positive patients (Ozyurt et al. 2023). MEF2C was also recognized as a target gene for miR-551a in breast cancer (Kang et al. 2019). The GO analy-

sis of the proposed target genes of miR-551a showed their involvement in critical biological processes, most importantly cell differentiation and circulatory system development. These findings suggest that the downregulation of miR-551a may lead to the upregulation of crucial genes involved in cardiac muscle development and differentiation, potentially contributing to cardiac dysfunction in breast cancer (Demissei et al. 2020; da Costa et al. 2021). The molecular function analysis of miR-551a's target genes revealed functions such as endopeptidase inhibitor activity and hyaluronan glucosaminidase activity, which have been associated with breast cancer progression (Sarka et al. 2023). KEGG pathway analysis showed that the downregulation of miR-551a dysregulates the Sphingolipid metabolism pathway, Mapk signalling pathway, Notch signalling pathway, and ErbB signalling pathway. Dysregulation of the Sphingolipid metabolism pathway is known to contribute to tumour growth in various cancers, including breast cancer (Companiononi et al. 2021; Pani and Dasgupta 2023). The Notch signalling pathway, critical for cell differentiation, has been aberrantly activated in breast cancer. Its implications extend to tumour initiation, progression, and breast cancer stem cell activity (Pandey et al. 2023). Mapk and ErbB signalling pathways are known to be overactivated in breast cancer (Wang 2017; Naik 2019). The PPI analysis shown that ERBB4 has been identified to connect with the NGR gene family. The connection between ERBB4 and the NGR family was reported previously and showed to be overexpressed in breast cancer and have been identified as diagnostic and therapeutic targets (Vulf et al. 2023). The bioinformatics analysis from this study pinpointed ErbB4 as the primary target gene of miR-551a within the Mapk and ErbB signaling pathways. Additionally, the interaction of ErbB4 with the NGR family further emphasizes the crucial role of miR-551a as a tumour suppressor in breast cancer, suggesting that its downregulation might lead to the upregulation of ErbB4. In conclusion, this study highlights that miR-551a is downregulated in breast cancer, leading to the dysregulation of several crucial signalling pathways in breast cancer. Furthermore, it suggests that miR-551a might target CRB2 and TMPRSS2, with ErbB4 being of particular significance.

The researchers observed a significant upregulation of miR-4534 in breast cancer tissues compared to normal tissues. To the best of the researcher's knowledge, miR-4534 has been consistently upregulated in all studies related to cancer. The observation in this research is consistent with previous findings on miR-4534 across various cancer types. For instance, extensive research on prostate cancer consistently supports this result. A study indicated that miR-4534 was upregulated in prostate cancer tissues, promoting prostate cancer angiogenesis by downregulating VASH1 (Jiang et al. 2020). Further supporting this consensus, another study emphasized the overexpression of miR-4534 in prostate cancer. Its oncogenic effects were attributed, in part, to the downregulation of the PTEN pathway. This suppression might enhance cell proliferation and survival in cancer cells, reflecting the oncogenic effects of miR-4534 as observed in the current research (Nip et al. 2016). Additionally, the upregulation of miR-4534 is evident in benign prostatic hyperplasia when compared to healthy controls. The study also reported association of increased miR-4534 expression with lymph node metastasis, highlighting its crucial role in cancer progression (Öztürk et al. 2022). Supporting the current research findings, in the context of colorectal tumorigenesis, miR-4534 was found to be upregulated in tissues deficient in p53. The study underscored miR-4534's role in regulating autophagy by suppressing ATG2B in the tumour stroma (Inoue et al. 2021). Similarly, another study using RNA sequencing identified miR-4534 as upregulated in triple-negative breast cancer, reinforcing the current findings. This research also identified miR-4534 as an upstream regulator of ADAMDEC1. Furthermore, the negative correlation of miR-4534 with immune markers offers a potential explanation for its unfavorable prognosis, aligning with the current research (Chai et al. 2023). The upregulation of miR-4534 across various cancer types underscores its potential as a critical biomarker in cancer progression and prognosis. For further exploration of miR-4534's role in breast cancer biology, bioinformatics analyses were performed and suggests SOX6, ADCY1, DAAM2, ESRRG, FOXQ1, and ZNF142 to be target genes for miR-4534. SOX6 is recognized for its pivotal role in organ development and cell differ-

entiation, including stem cell formation. Previous studies have pointed to the tumour suppressive functions of SOX6, especially in breast cancer, and reported to be downregulated in breast cancer (Wei et al. 2022). Also, ADCY1 is promoter-methylated and downregulated in breast cancer. There is a significant association between the elevated mRNA levels of ADCY1 and favorable outcomes in breast cancer patients (Li et al. 2017). DAAM2 has been reported to be downregulated in pancreatic adenocarcinoma (Zhang et al. 2022). ESRRG has been identified as a tumour suppressor and shown to be downregulated in breast cancer. Its mechanism involves inhibiting glycolysis and bolstering oxidative phosphorylation (Eichner et al. 2010). Moreover, a low expression of FOXQ1 has been reported to be associated with poorer overall survival in breast cancer, rendering it a crucial factor in prognosis (Elian et al. 2021). Finally, ZNF142 stands out with a mutation burden reported in over a quarter of breast cancer samples (Aravind et al. 2018; Yan et al. 2021). The results suggest that miR-4534 is upregulated in breast cancer downregulating SOX6, ADCY1, DAAM2, ESRRG, FOXQ1, and ZNF142. Through GO analysis, the research noted that the miR-4534 suggested target genes were enriched in regulating cation channel activity, protein complex oligomerisation, and regulation of sodium ion transmembrane transport, among other things. Changes in ion channels, specifically those on cation channel activity, have been linked to malignancies, including breast cancer (Jiang et al. 2021). The KEGG pathway analysis underscores the influence of miR-4534 on the PI3K-Akt, glutamatergic synapses, cholinergic synapses, and relaxin signalling pathways. PI3K-Akt signalling pathway has been previously identified as a target pathway for miR-4534 (Zhao et al. 2020). Noteworthy, the dysregulation of glutamatergic synapse, and cholinergic synapse signalling pathways are known to impact cell growth, tumour proliferation, and endocrine resistance in breast cancer. It is also essential to note the pivotal role that ion channels play in the functioning of the glutamatergic and cholinergic pathways (Willard and Koochekpour 2013; Jiang et al. 2021; García et al. 2022).

The PPI analysis showed that ADCY1, the target gene of miR-4534, interacts with the adenylate cyclase and G-protein families, which oversee a plethora of cellular processes linked with tumorigenesis, angiogenesis, and metastasis. Importantly, this research demonstrates that ADCY1 is a target gene in glutamatergic and cholinergic synapse signalling pathways (Fan et al. 2019; Zou et al. 2019).

Consequently, the study pinpointed that upregulated miR-4534 might be oncogenic and contribute to the progression of breast cancer by perturbing the intricate network of different signalling pathways, most importantly the glutamatergic synapses and cholinergic synapses signalling pathways. Furthermore, its influence on various genes, most notably the ADCY1 target gene, was previously reported. While our results underscore the potential therapeutic implications of modulating miR-4534 levels in managing breast cancer, they also highlight the need for further investigations, especially in vivo research, to confirm the target genes and pathways of miR-4534.

CONCLUSION

In conclusion, this study provides valuable insights into the roles of miRNAs such as miR-1294, miR-551a, and miR-4534 in breast cancer. The observed downregulation of miR-1294 and miR-551a in breast cancer tissues underlines their potential as tumour suppressors. Notably, the study suggests that miR-1294 targets genes such as BRCC3, MYCL, and YOD1, which are significantly involved in the Hippo and p53 signalling pathways. Also, miR-551a, also found to be downregulated in breast cancer, potentially targets ERBB4, MEF2C, CRB2, and TMPRSS2. These genes play key roles in various pathways, including cardiac muscle regulation, Sphingolipid metabolism, Notch signalling, Mapk signalling, and ErbB signalling. The study indicates an oncogenic role for miR-4534 due to its upregulation in breast cancer. It targets genes involved in ion transport, glutamatergic and cholinergic synapse signalling pathways, thus underscoring its potential as a therapeutic target. The study suggests genes such as SOX6, ADCY1, ESRRG, and ZNF142 as potential targets of miR-4534.

RECOMMENDATIONS

The insights gleaned from this study offer a robust starting point for researchers to delve further into investigating the suggested target genes in a laboratory setting. Although the bioinformatics analysis carried out in this study has proven invaluable, experimental validation remains essential for the identified target genes potentially regulated by miR-1294, miR-551a, and miR-4534. Extending these investigations to in vivo models, notably mouse models, will permit the examination of how these miRNAs and their target genes influence tumour growth, metastasis, overall survival, and their role in the tumour microenvironment. Future studies should consider expanding the sample size and including samples with distinct pathological characteristics such as metastasis, tumour size, and cancer biochemical markers. This will allow for a comprehensive evaluation of the relationship between the studied miRNAs and clinicopathological factors, thereby facilitating a better understanding of the biological functions of these miRNAs and their influence on breast cancer.

LIMITATIONS

The execution of this study during the COVID-19 pandemic presented inherent limitations. One key restriction was the reduced sample size, as a considerable number of potential participants avoided hospital visits due to the health crisis. Additionally, financial constraints prevented the researchers from conducting laboratory validations of the target genes for the studied miRNAs. As a result, the researchers relied solely on bioinformatics analyses. This dependence on a limited sample and the lack of experimental validation may have influenced the strength of the findings. Future investigations should address these limitations, thereby providing a more comprehensive and accurate understanding of these miRNAs and their functions.

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ETHICAL CLEARANCE

The work titled “miRNA Expression analysis in Breast Cancer” was presented to the ethics committee in Gaziantep University at the faculty of biology/molecular biology department and approved on 09/01/2019. The Committee’s operation basis and the name and title of the president of the are given below. Full list of committee members is in the original Ethics Committee Report in Turkish.

CLINICAL RESEARCH ETHICAL COMMITTEE

The working basis of the ethic committee was the Regulation on Clinical Trials of Drugs and Biological Products and Good Clinic application guideline.

President: Prof. Dr. Aysun BARANSEL ISIR

NOTES

- 1: All the histological and biochemical analysis results were obtained from the hospital data.
- 2: N1 (one or more lymph nodes or underarms)
- 3: N2 (internal mammary lymph nodes)
- 4: N3 (widespread)
- 5: CEA normal reference: From 0 to 2,5 µg/L
- 6: CA-125 normal reference: From 0 to 35
- 7: CA15-3 normal reference: 30 U / mL
- 8: Ki-67 is a prognostic parameter in breast cancer patients
- 9: Grade G1 (Ki-67 index is lower than 2%)
- 10: Grade G2 (Ki-67 index is from 3 to 20%)
- 11: Grade G3 (Ki-67 index is more than 20%)

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