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Deciphering the prognostic impact of aberrant DNA methylation on *ANGPT1* gene in breast cancer

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Breast cancer (BC) is a multifaceted disease distinguished by a range of molecular subtypes and varying clinical prognoses. The involvement of DNA methylation in the dysregulation of gene expression has been linked to the development and progression of BC. Therefore, this study aimed to investigate the association between *ANGPT1* gene expression and DNA methylation in BC patients. Eight Saudi female blood samples were used to undergo for whole genome bisulfite sequencing (WGBS) and RNA sequencing for the identification of novel DNA methylation targets. Several public domain BC datasets including the METABRIC cohort, TCGA, and Kaplan Meier Plotter datasets, were used to explore the prognostic significance of *ANGPT1* gene. Then, the demethylation agent 5-aza-2′-deoxycytidine was used to examine the potential association between DNA methylation and *ANGPT1* expression. Finally, the validation was conducted on blood samples from 49 Saudi females using methylight techniques. Our results shows that upregulation of *ANGPT1* gene expression exhibited hypomethylation pattern in BC samples. these results were confirmed by MCF7 cell line experiments. Demethylating using 5-aza in MCF7 and MCF10A showed a high expression of *ANGPT1* in both cell lines. *ANGPT1* mRNA expression was found to poor prognostic biomarker and lower Breast Cancer-Specific Survival (BCSS) in BC patients. The potential importance of abnormal DNA methylation in the development and advancement of BC is significant. *ANGPT1* may act as an oncogene and could be extensively studied further to behave as a predictive biomarker for breast cancer.

Keywords Breast cancer, DNA methylation, Biomarker, ANGPT1, 5-aza-2'-deoxycytidine

Breast cancer (BC) is a common form of cancer that is frequently diagnosed among women both globally and in Saudi Arabia. According to data from 2018, Out of the total cancer cases in Saudi Arabia, 29.7% of them were breast cancer cases in the female population[1]. The incidence of BC in the female population of Saudi Arabia has demonstrated a significant increase of over the course of 16 years, rising from 545 cases in 2001 to 2463 cases in 2017[2]. The progress and metastasis of BC are deemed to be the result of not only genetic abnormalities (such as *TP53*, *PIK3CA* mutation, and *BRCA1/BRCA2* inactivation) but also alterations in epigenetic factors[3] The occurrence of irregular DNA methylation is a prevalent epigenetic modification observed in various types of cancer, such as BC. Nonetheless, the current research on DNA methylation in BC among Saudi females is

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constrained[4] The process of methylation seems to occur at an early stage in the development of BC, leading to the activation of multiple oncogenes and the suppression of tumor suppressors, which encourages the proliferation of abnormal cells[5].

Angiopoietin-1 Ang1 (*ANGPT1*) is an oligomeric glycoprotein and a representative of the angiopoietin family of growth factors, along with Ang2 and Ang3/4. It is located at chromosome 8q23.1. The *ANGPT1* is necessary for the proper organizing and angiogenesis of capillaries from the vasculature that already exists as it supports the quiescence and structural stability of the adult vasculature. In addition, *ANGPT1* possesses a powerful effect on mature vessels, involving enhancement of vessel survival, reduction of inflammatory gene expression, and control of vascular leakage[6]. In addition to VEGF, Angiopoietins (*ANGPT-1* and *ANGPT-2*) and its receptor tyrosine kinas) Tie-2(have been identified as significant factors in the initiation of angiogenesis. *ANGPT-1* is the primary molecule that triggers the activation of the Tie-2 receptor by phosphorylation. This activation supports the survival of endothelial cells and helps in stabilizing blood vessels[7]. Angiogenesis, a characteristic feature of human malignancies, is one of the main factors contributing to tumor growth. Angiogenesis refers to the process in which cancer cells consume oxygen and nutrients, and the distance for substances to diffuse across the stroma between a nearby capillary and the demanding cell is limited to approximately 100 mm. By altering the balance of angiogenic promoters and inhibitors, the tumor generates capillary grows in the surrounding blood vessels[8] Although *ANGPT1* can promote the infiltration of immune cells, it also plays a dual role in the advancement of tumors. Lower levels of *ANGPT1* expression have been linked to improved patient prognosis in some situations, such as endometrial cancer. This suggests that increased expression of *ANGPT1* may be related with negative outcomes. This can be ascribed to its function in encouraging the formation of new blood vessels (angiogenesis) and perhaps aiding in the spread of tumors and their growth in other parts of the body (metastasis)[9] .Moreover, *ANGPT1* was discovered to be a prognostic factor associated with immune infiltration. The expression of *ANGPT1* was observed to have a substantial correlation with overall survival. This suggests that *ANGPT1* could be a new prognostic marker for the immunological microenvironment of lung cancer and a potential target for immunotherapeutic treatments[10].

Moreover, it is a crucial determinant for the development of blood vessel maturity. By attaching to the Tie-2 receptor, *ANGPT1* influences the signaling pathways of PI3K/AKT and MAPK/ERK, which play a crucial role in regulating the development, proliferation, and survival of endothelial cells[11]. The expression levels of *ANGPT1* in breast cancer can exhibit variability, and its function may vary depending on the tumor microenvironment and molecular subtype. Several studies have suggested that increased levels of *ANGPT1* may be associated with unfavorable outcomes, serving as an unfavorable biomarker. In certain settings, decreased expression levels of *ANGPT1* have been linked to more favorable prognostic outcomes in breast cancer, indicating the intricate nature of its activity[12]. Although there is existing data indicating a correlation between *ANGPT1* and DNA methylation in several cancers, additional investigation is required to comprehend the ramifications and mechanisms underlying this association comprehensively. In addition, based on the available literature, it seems that there a gap directly linking *ANGPT1* expression and incidence of BC angiogenesis and progression, especially in Saudi Arabia. The purpose of this study was to analyze the gene expression and methylation level of *ANGPT1* in Saudi BC patients.

Results

Demographic characteristics for BC patients

This study included a sample size of 49 females from Saudi Arabia, consisting of 28 individuals diagnosed with BC and the other participants acting as normal. The predominant tumor type seen in the patient population was invasive ductal carcinoma (IDC), accounting for 89.3% of cases, while invasive lobular carcinoma (ILC) constituted 10.7% of cases. The analysis of familial lineage revealed that most patients (64.3%) lacked any documented instances of cancer within their family history. Conversely, the remaining 35.7% of patients did possess a familial predisposition to cancer. In terms of molecular classification, it was shown that 71.4% of the patients exhibited Lumina A subtype, while the other subtypes, namely Lumina B, Lumina B like HER2-enriched, and Triple-negative, each accounted for 7.1% of the patient population as shown in (Table 1).

Further analysis of the data used two analyzed databases, the Molecular Taxonomy of BC International Consortium (METABRIC) ($n=1980$) and The Cancer Genome Atlas (TCGA) ($n=854$). The demographic of participants utilizes several characteristics including age, tumor size, tumor grade, lymph node stage, lymph vascular invasion, PAM50 subtype, hormonal receptors[13].

Methylation level differences and the distribution of hyper- and hypo-methylated regions among samples

The heatmap (Fig. 1) displays the findings of Pearson's correlation analysis of methylation levels between samples. Nevertheless, most of the sample pairs exhibit robust positive correlations, with values predominantly ranging from 0.936 to 1. The data clearly indicates that N1 has exceptionally strong correlations (exceeding 0.95) with BC samples.

Moreover, the whole genome bisulfite sequencing (WGBS) results revealed 7406 differentially methylated regions (DMRs), the DMRs were identified using the DSS software (Hao Feng HaoWu, 2014; Hao Wu, 2015; Yongseok Park Hao Wu,2016), The core of DSS is a new dispersion shrinkage method for estimating the dispersion parameter from Gamma-Poisson or Beta-Binomial distributions, the hypomethylated regions represents 5107 (68%) and hypermethylated regions represents 2299 (31%). For DMR distribution, the highest distribution of hyper- and hypomethylation was observed in intron regions then promoter, exon, 3 UTR, and 5 UTR as shown in (Fig. 2).

	Valid	Frequency	Percentage %
Age	< 50	10	35.7
	≥ 50	18	64.2
Weight	58–80	18	64.3
	81–102	10	35.7
Tumor stage	0	2	7.1
	1	8	28.6
	2	14	50.0
	3	4	14.3
Tumor type	IDC	25	89.3
	ILC	3	10.7
Family history	NO	18	64.3
	YES	10	35.7
Treatment	NO	11	39.3
	YES	17	60.7
Molecular type	Lumina A	20	71.4
	Lumina B	2	7.1
	Basal like	2	7.1
	HER2-enriched	2	7.1
	Triple-negative	2	7.1

Table 1. The demographic information of BC patients.

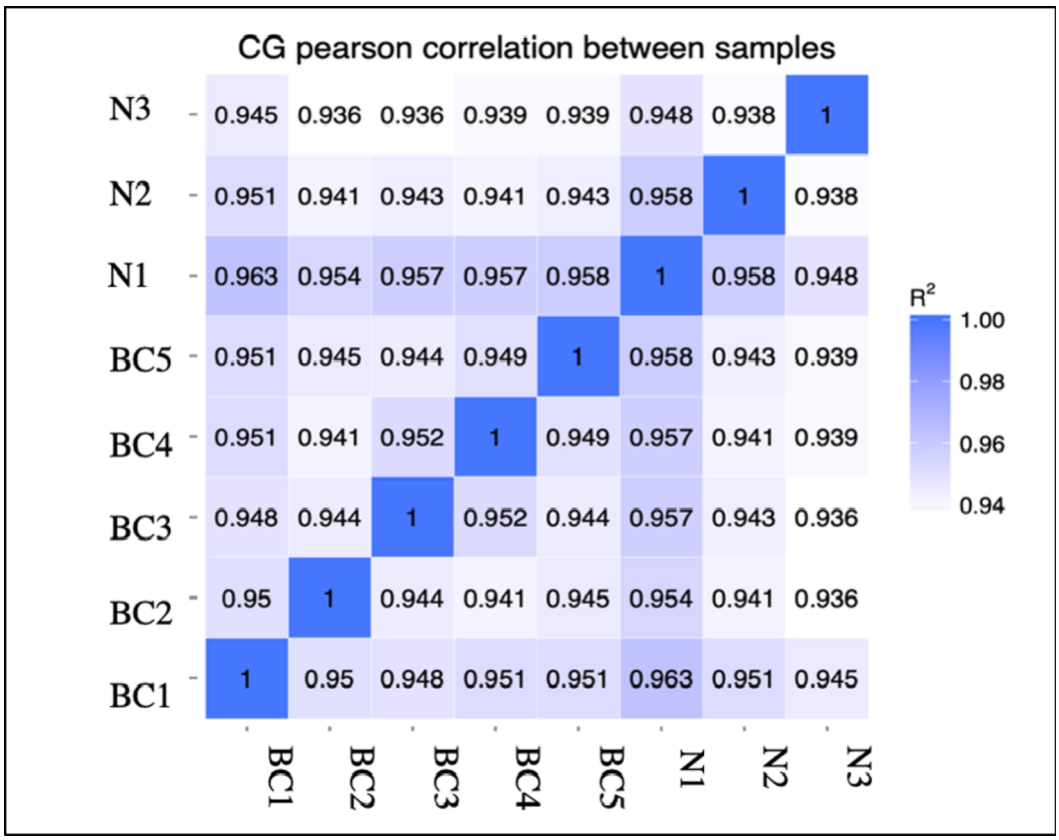

Fig. 1. Correlation analysis of DNA methylation. The correlation of methylation levels between samples is an important indicator to test the reliability of the experiment and whether the sample selection is reasonable. The closer the correlation coefficient is to 1, the higher the similarity in the methylation pattern between samples. Whereas BC1,2,3 reveals to breast cancer patients and N1,2,3 reveals to normal samples. R^2: the square of person correlation (0.94 to 1).

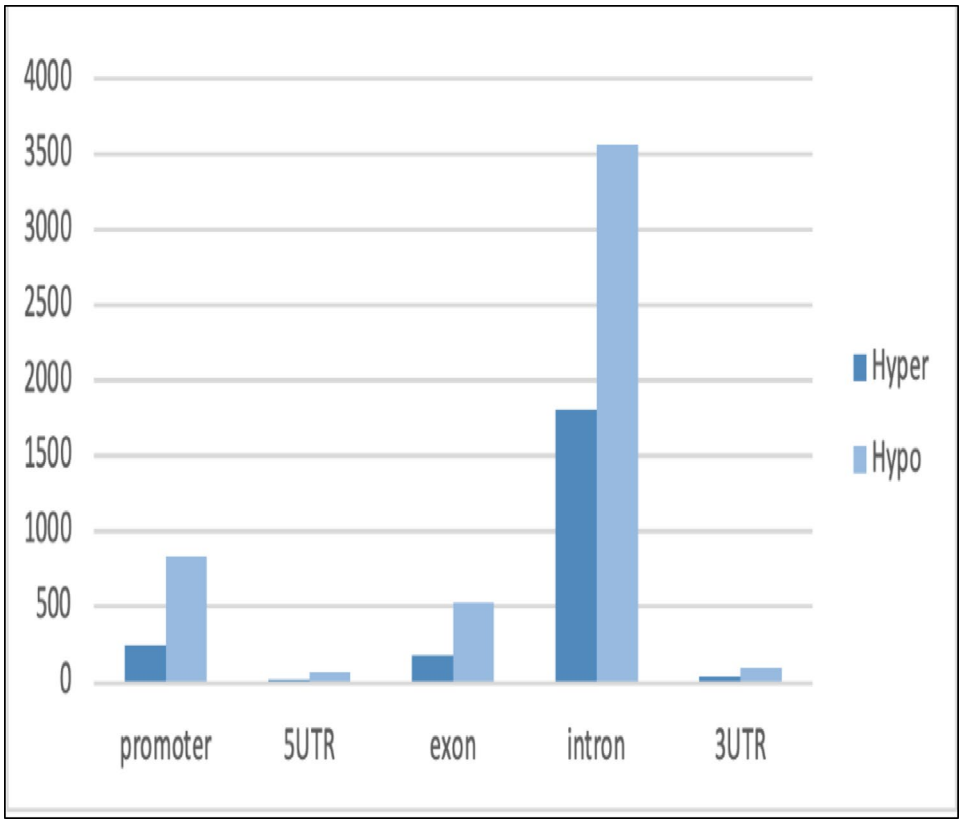

Fig. 2. DMR distribution plot in CG. For DMR target regions (such as promoter, exon, intron, CGI, CGI shore, repeat, TSS, TES, etc.), we provide statistics between hyper (high methylation DMR) and hypo (low methylation DMR). The x-axis is functional regions, the y-axis is number of hyper/hypomethylated regions.

Evaluation of gene expression levels across samples and identification of key differentially expressed genes

The DESeq2 R package (1.20.0) was utilized for the differential expression analysis of the samples. The Pearson's correlation results of gene expression between breast cancer (BC) and normal samples were displayed in the heatmap (Fig. 3). The BC group had a strong association, with an average R2 value of at least 0.92. In contrast, the normal group showed a weaker correlation, with an R2 value of at least 0.61.

In addition, the RNA sequencing data showed that 1912 genes had differential expression in BC samples. Among these genes, 1117 (83%) were upregulated, whereas 795 (17%) were downregulated. The most upregulated genes are *ZNF292*, *DGKH*, *CCDC186*, and *ANGPT1*. Furthermore, the most downregulated genes are *AC099778.1*, *RP9*, *TPPP3* and *PCP2*. However, genes with P-value ≤ 0.05 were considered as differentially expressed (Fig. 4).

ANGPT1: a key hypomethylated gene with altered expression in the MAPK pathway of BC patients

The analysis showed four overlapped aberrant hypomethylated genes *(ANGPT1*, *CACNA2D3*, *FLNA*, *and IKBKG)* with the MAPK pathway genes (Fig. 5). The human whole genome bisulfite sequencing data indicated that the most significant difference between the BC patients and normal samples of methylation level was found for the *ANGPT1* gene. The methylation level in normal samples was 0.814, whereas in BC patients it was 0.371 with a P value of 6.78E−10. Nevertheless, the RNAseq analysis revealed that the *ANGPT1* expression level in the control group was 4.03, whereas in the BC group, it was 201.10 as shown in (Table 2).

The clinicopathological characteristics of *ANGPT1* expression

(Table 3) presents a concise overview of the clinicopathological features in BC patients exhibiting high expression levels of *ANGPT1*. Based on the findings from the METABRIC study ($n = 1980$) and the TCGA datasets ($n = 854$). The expression was correlated with LVI, including tumor grade, hormone receptor [ER and PR], and HER2. In the METABRIC cohort, a statistically significant high *ANGPT1* was observed in patients with histological as well as grade 3, negative (ER, PR, HER2), and non-TNBC. In TCGA cohort, a statistically significant high *ANGPT1* was observed in patients with positive (ER, PR) negative HER2 and non-TNBC.

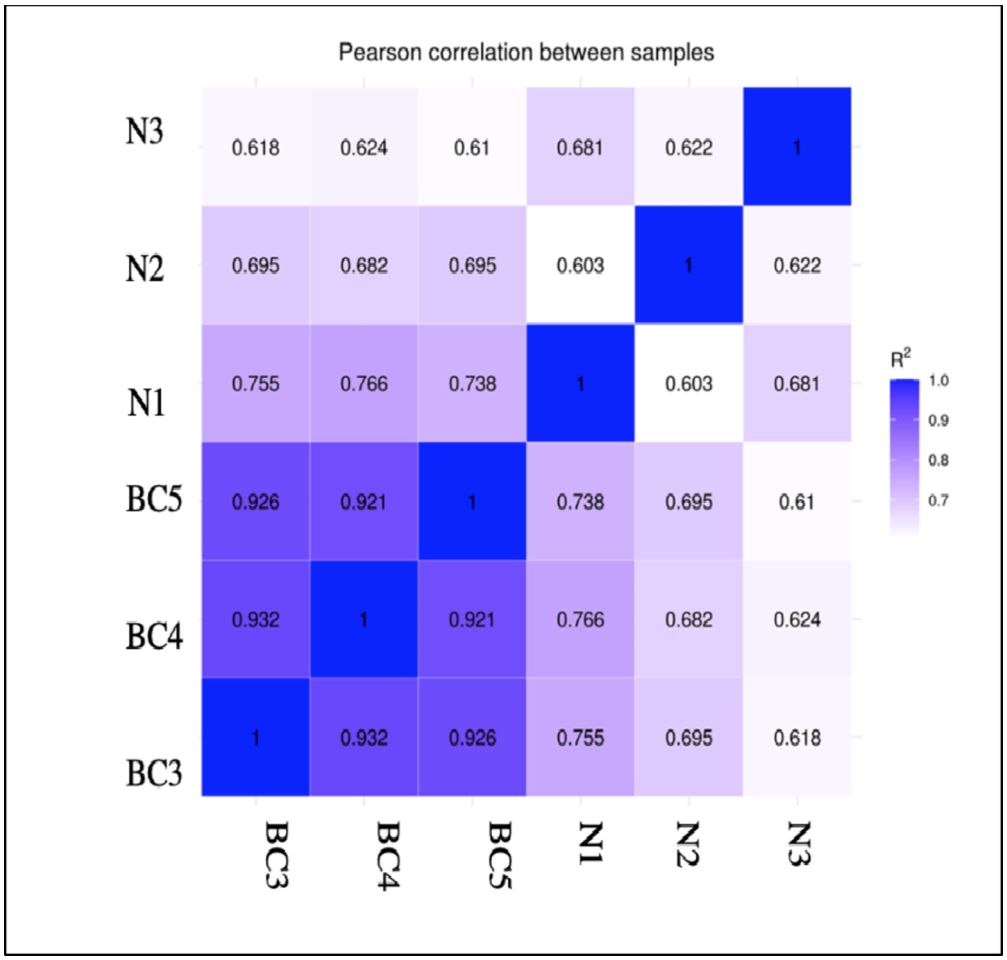

Fig. 3. Correlation analysis of RNAseq. According to all gene expression levels (RPKM or FPKM) of each sample, the correlation coefficient of samples between groups is calculated and drawn as heat maps. It is intuitive to show sample differences and repeat cases between groups. The higher the correlation coefficient of the sample is, the closer the expression pattern is. Whereas BC1,2,3 reveals to breast cancer patients and N1,2,3 reveals to normal samples. R2: the square of person correlation (0.7-1).

Induced global DNA methylation by 5-aza-2′-deoxycytidine increases the expression of *ANGPT1* in BC patients

From cell culture experiment, In the MCF7 BC cell line, the *ANGPT1* was represented as a high expression level compared to its expression in the MCF10A normal breast cell line (Fold Change = 1.3). To assess the impact of methylation on *ANGPT1* gene expression, the 5mM of 5-aza-2'-deoxycytidine was applied to induce genome-wide demethylation by specifically targeting DNA methyltransferases (DNMTs). This compound incorporates into the DNA of dividing cells, forming covalent bonds with DNMTs and inhibiting their activity. The effectiveness and rate of demethylation are closely linked to the cell's replicative rate and the duration of treatment, as the incorporation of 5-aza-2'-deoxycytidine into DNA occurs during the S-phase of the cell cycle. MCF7 and MCF10A cell lines were treated with this compound, allowing us to compare the *ANGPT1* expression levels before and after three and seven days of the treatment with 5-aza. As a result, the *ANGPT1* expression levels were significantly high in both cell lines treated with the 5-aza compared to untreated cell lines. In the MCF7 cell line, *ANGPT1* expression levels increased significantly, showing a fold change of 2.8 on the third day compared to the untreated group and a dramatic rise to a fold change of 16.38 by the seventh day. Similarly, in the MCF10A cell line, *ANGPT1* expression levels also increased, with a fold change of 4.89 observed on the third day and 6.35 on the seventh day, as illustrated in (Fig. 6).

Methylight qPCR result

Methylight quantitative PCR was employed to assess the methylation level of the first exon of *ANGTP1*. Following the process of bisulfate conversion. Interestingly, there were no statistically significant differences in the methylation level seen between individuals with BC and the control group at the CpG sites targeted by the *ANGTP1* probe in the first exon (Fig. 7).

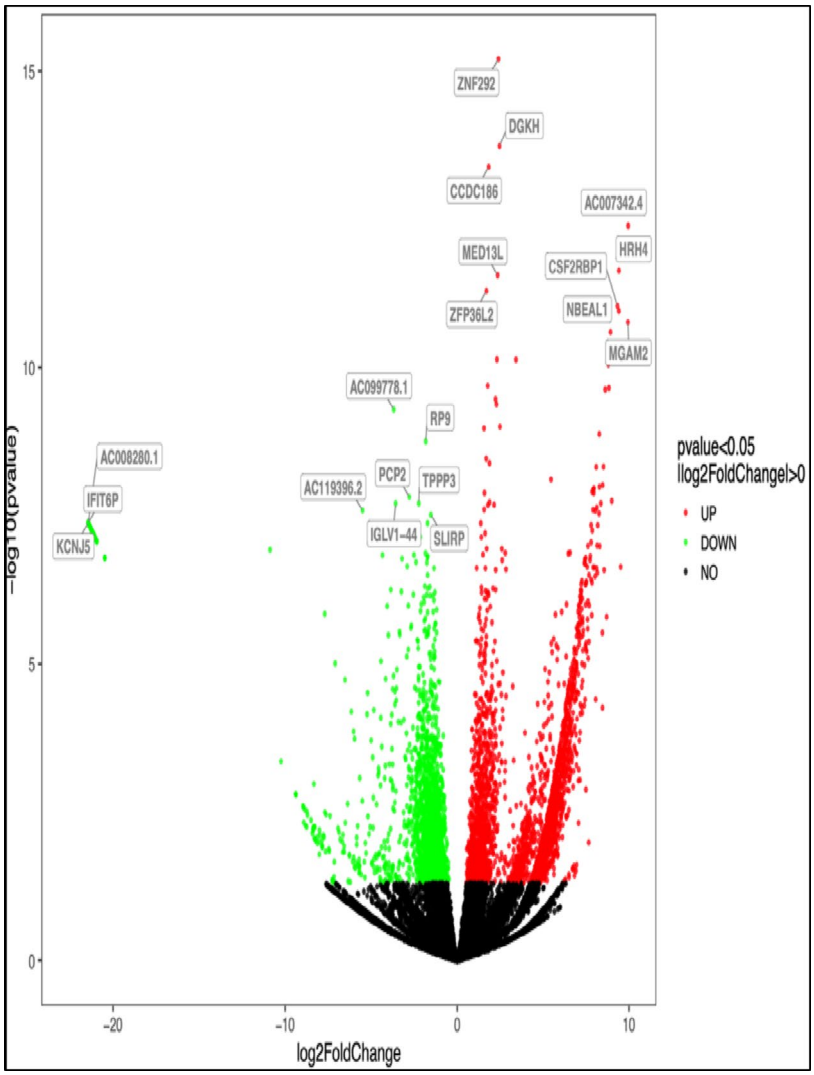

Fig. 4. Volcano map of differentially expressed genes in BC patients. Volcano plots can be used to infer the overall distribution of differentially expressed genes. In the figure, the x-axis shows the fold change in gene expression between different samples, and the y-axis shows the statistical significance of the differences. Red dots represent up-regulation genes and green dots represent down-regulation genes.

Gene expression analysis of *ANGPT1*

In order to validate the observed differences in gene expression levels, quantitative polymerase chain reaction (qPCR) was used. The qPCR experiment was performed in triplicate for the selected genes. The qPCR expression values for the *ANGTP1* gene were normalized using the housekeeping gene *GAPDH*, which exhibited comparable ex-pression levels across all BC samples. The t-test was used to evaluate the levels of ex-pression between samples of BC and normal samples. A total of 24 samples were analyzed for this experiment. The present investigation revealed a statistically significant increase in the expression of *ANGPT1* among individuals diagnosed with breast cancer, a P-value of 0.04. as shown in (Fig. 8).

Evaluation of *ANGPT1* in BC: expression and promoter methylation patterns from TCGA and TNMplot

The UALCAN website conducted an analysis on a dataset consisting of 1097 primary tumor samples of BC and 114 normal samples obtained from the Cancer Genome Atlas (TCGA). The findings of this analysis were statistically significant, as indicated by a p-value of less than 0.01. In BC samples, the median expression levels were found to be 0.934 transcript per million. As shown in (Fig. 9).

In a separate examination, the methylation status of the promoter region of the *ANGPT1* gene was assessed in a total of 793 primary tumor samples and 79 normal samples sourced from the TCGA database. The findings of the study indicated the Beta value for the level of DNA methylation of BC and normal samples was 0.723 and 0.673 respectively. This finding is illustrated in (Fig. 10).

Moreover, the TNMplot page was used to analyze the expression of *ANGPT1* in a total of 113 normal samples, 1097 tumor samples, and seven metastatic tissue samples. The findings presented in the study demonstrated a

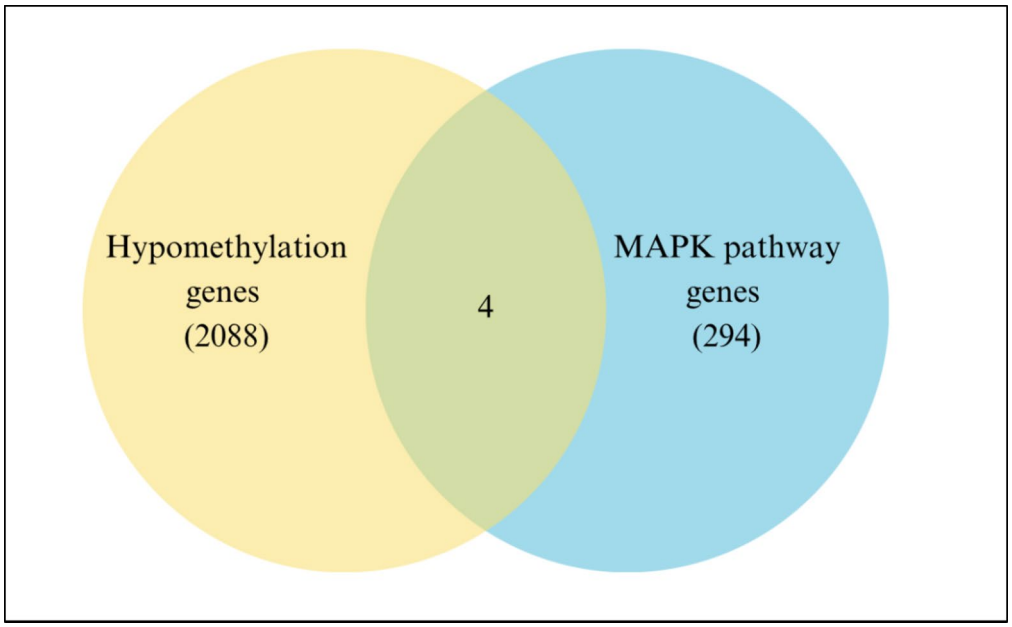

Fig. 5. Veen diagram of overlapped MAPK pathway genes and hypomethylated genes. The category ratio is the enrichment ratio (for ORA), indicating the gene ontology sets with FDR < 0.05. The category p-value indicates the weighted set cover and maximum coverage called size-constrained weighted set cover, where weights are assigned to gene sets with smaller enrichment p-values.

Gene name	*N* methylation	BC methylation	*P* value	*N* DEG	BC DEG	*P* value	FC
ANGPT1	0.814	0.371	**6.78E−10**	4.031	201.100	**0.0002**	5.56

Table 2. Methylation and expression levels in ANGPT1. Significant values are in bold.

notable disparity in the expression of *ANGPT1* between normal-tumor tissues and tumor-metastatic tissues. The statistical analysis revealed p-values below 0.01 for both comparisons, indicating a statistically significant difference. This result is shown in (Fig. 11).

The correlation between *ANGPT1* expression and breast cancer-specific survival (BCSS) rate

In the METABRIC and KM-Plotter datasets the outcome analysis of an association between high *ANGPT1* mRNA expression and shorter BCSS ($p = .0.042$, HR = 1.389, 95% CI = 1.012–1.905) (Table 4). The study of outcomes in the METABRIC and KM-Plotter datasets revealed a significant correlation between elevated *ANGPT1* mRNA expression and reduced BCSS. including Her2 Expression ($p < 0.001$, HR: 1.601, 95% CI 1.218–2.103), Tumor Size ($p = .0.006$, HR: 1.440, 95% CI 1.112–1.865), Lymphovascular Invasion ($p = .008$, HR: 1.364, 95% CI 1.086–1.713), and Lymph Node Stage ($p < .001$, HR: 1.762, 95% CI 1.524–2.038). The examination of the KM plotter datasets indicated that BC patients ex-hibiting high *ANGPT1* expression exhibited a poor BCSS ($p = .0.0759$, HR: 1.23; 95% CI 0.98–1.53) (Fig. 12).

Discussion

DNA methylation is a dynamic phenomenon characterized by the ability to undergo temporal alteration that affects gene expression. The alteration of the expression of certain genes in BC is closely related to DNA methylation. DNA methylation changes are often seen in BC and are known to arise in the early stages of the development and progression of the disease[14] The process of angiogenesis is of utmost importance in the initiation and progression of tumorigenesis, since solid tumors need a vascular network to surpass a limited size of a few millimeters. Angiogenesis refers to the process through which new blood and lymphatic vessels are generated from pre-existing vasculature. This enables neoplastic cells to get nourishment in the form of essential nutrients and oxygen, as well as the capacity to eliminate metabolic waste products[15]. The *ANGPT1* belongs to the family of growth factors called angiopoietins, which also include Ang2 and Ang3/4. Angiopoietin-1 is a protein that plays a crucial role in regulating blood[16].

Global hypomethylation is a widespread occurrence reported in various forms of cancer. This has the potential to induce chromosomal instability, reactivate transposable elements, and increase mutation rates. In addition, the process of hypomethylation may induce the activation of oncogenes, potentially promoting the development of carcinogenesis. The current investigation observed a decline in the total DNA methylation level in the genome of BC samples. This discovery strongly corroborates the findings of previous research in

Parameters	METABRIC cohort $n=1980$			TCGA cohort $n=854$		
	Low	High		Low	High	
	N (%)	*N* (%)	*p* value	*N* (%)	*N* (%)	*p* value
Tumor size						
≤ 2.0 cm	555 89.4%	66 10.6%	0.816	58 38.7%	92 61.3%	0.847
> 2.0 cm	1185 88.9%	148 11.1%		182 39.9%	274 60.1%	
Nodal status						
Negative	897 90.6%	93 9.4%	0.054			
Positive	799 87.8%	111 12.2%				
Lymph node stage						
1	893 90.8%	91 9.2%	0.053	7 53.8%	6 46.2%	0.493
2	540 87.0%	81 13.0%		269 38.1%	437 61.9%	
3	284 89.9%	32 10.1%		52 39.7%	79 60.3%	
Histological grade						
Grade 1 and 2	824 89.0%	102 11.0%	0.616	196 35.3%	360 64.7%	0.802
Grade 3	571 89.9%	64 10.1%		132 44.9%	162 55.1%	
Lymphovascular invasion						
Negative	824 89.0%	102 11.0%	0.616	196 35.3%	360 64.7%	0.008
Positive	571 89.9%	64 10.1%		132 44.9%	162 55.1%	
Estrogen receptor						
Negative	345 72.8%	129 27.2%	**< 0.001**	52 28.3%	132 71.7%	**< 0 0.001**
Positive	1414 94.3%	86 5.7%		262 41.2%	374 58.8%	
Progesterone receptor						
Negative	767 81.8%	171 18.2%	**< 0.001**	86 31.7%	185 68.3%	**< 0 0.001**
Positive	992 95.8%	44 4.2%		228 41.6%	320 58.4%	
Human epidermal growth factor receptor 2						
Negative	1528 88.5%	199 11.5%	**< 0.001**	222 38.1%	360 61.9%	0.446
Positive	231 93.5%	16 6.5%		50 37.0%	85 63.0%	
Triple negative breast cancer status (TNBC)						
TNBC	205 64.1. %	115 35.9%	**< 0.001**	32 27.1%	86 72.9%	**0.008**
Non TNBC	1554 94.0%	100 6.0%		283 40.3%	420 59.7%	

Table 3. Association of ANGPT1 mRNA expression with clinicopathological characteristics in the METABRIC ($n=1980$) and TCGA ($n=854$) datasets. Significant correlations are in bold.

this field, which have established a connection between aberrant DNA methylation, a common occurrence in epigenetic changes, and several forms of cancer, including breast cancer (BC)[17–19]. Another finding of this study is that DMR (Differentially Methylated Region) had a high degree of distribution inside intron areas, followed by promoter regions, and finally in exon regions. Introns, although being non-coding areas, contain regulatory elements like as enhancers, silencers, and regions essential to alternative splicing. These elements play a role in regulating gene expression and can be indirectly affected by changes in intron methylation. Meanwhile, a silenced gene is deactivated through promoter hypermethylation, while gene activation can occur through promoter hypomethylation. Changes in exon methylation can impact the process of transcription elongation and splicing[20,21].

Differentially expressed genes (DEGs) are genes whose expression levels exhibit significant variation in illness samples compared to normal ones. This study found a substantial difference in the expression levels of

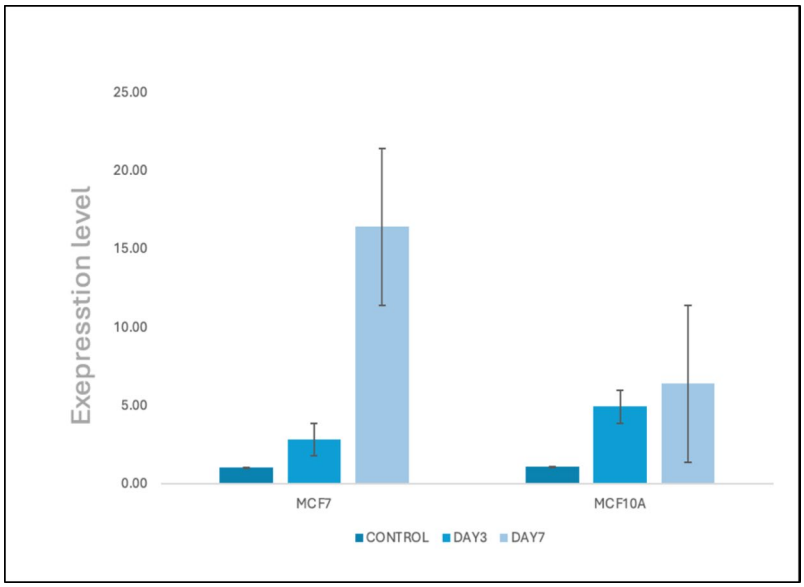

Fig. 6. The demethylating effect of 5-aza on ANGPT1 expression. Both cell line MCF7 and MCF10A exposures to 5-aza in day 3 and day 7.

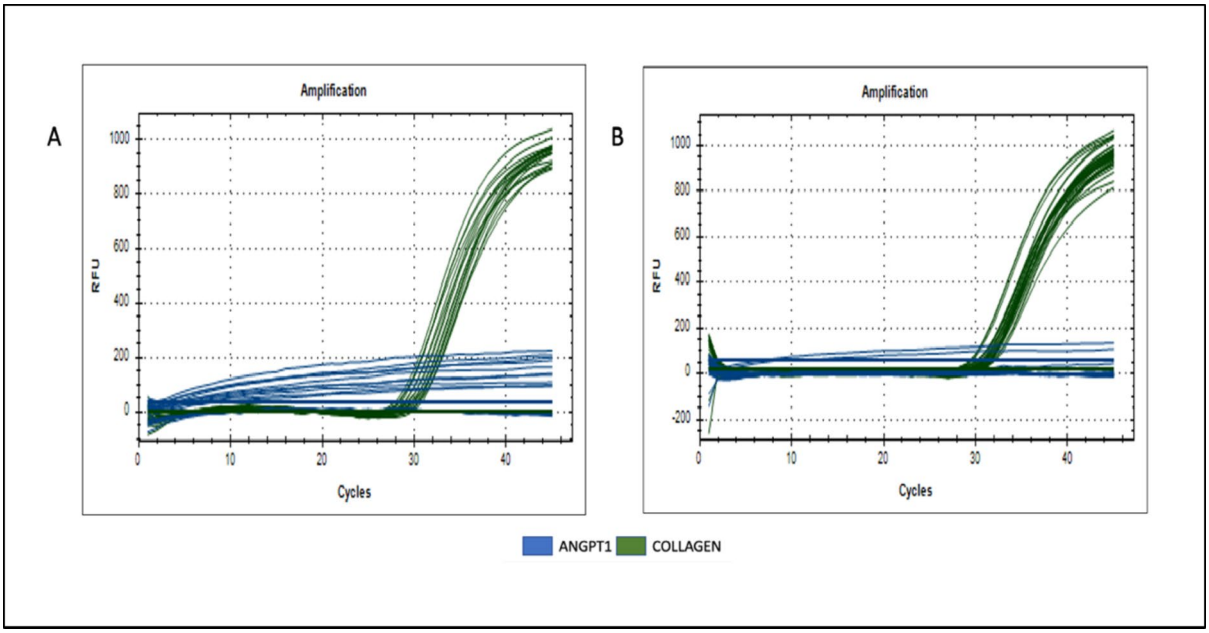

Fig. 7. The amplification plots of the *ANGTP1* and collagen probes. (**A**) BC patients (**B**) Normal samples. The *ANGPT1* gene is represented by blue color, while the reference gene is represented by green color.

1912 genes between BC samples and a normal group. Out of the differentially expressed genes (DEGs), 1117 (83%) showed upregulation whereas 795 (17%) showed downregulation. A recent study utilized transcriptome sequencing data to identify 937 differentially expressed genes (DEGs) in the BC samples. Among these DEGs, 487 genes were upregulated, and 450 genes were downregulated[22].

The results of our study using RNA sequencing and WGBS techniques revealed high expression and hypomethylation of *ANGPT1* gene in BC patients, this was also observed in several public data used in this study for examples TNM-plot (https://www.tnmplot.com/) and UALCAN (http://ualcan.path.uab.edu/index.html). The observed phenomenon is speculated to be explained by the hypothesis that the differential expression is influenced by the degree of methylation. Another important finding was found when using the UALCAN website to analyze the methylation levels in the promotor region of the *ANGPT1* gene in both cohorts (p-value < 0.01). Prior studies noted the importance of the occurrence of DNA methylation through the promoter region of tumor suppressor genes during the first phases of carcinogenesis. Decreasing methylation within the promoter

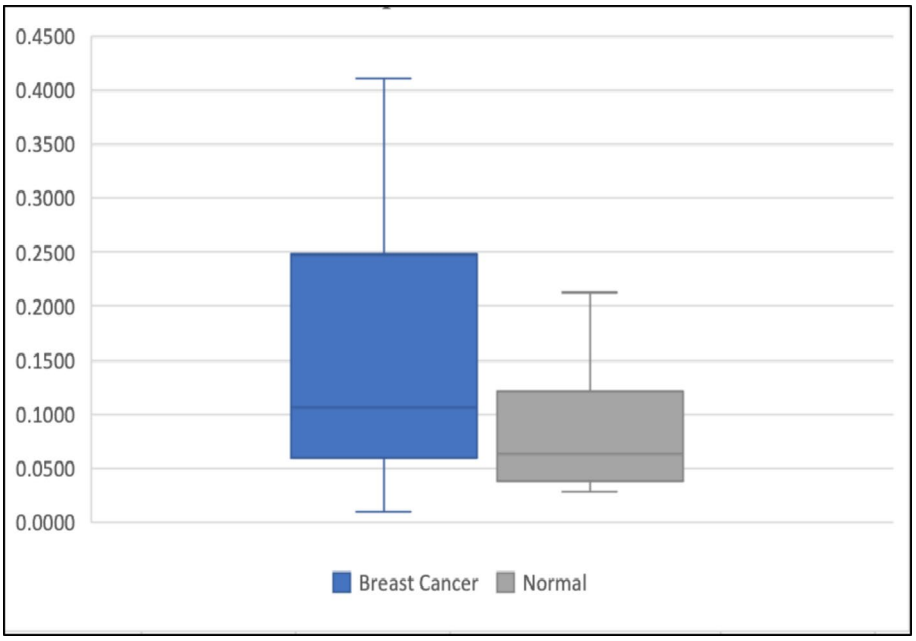

Fig. 8. The expression level of ANGPT1 between BC patients and normal.

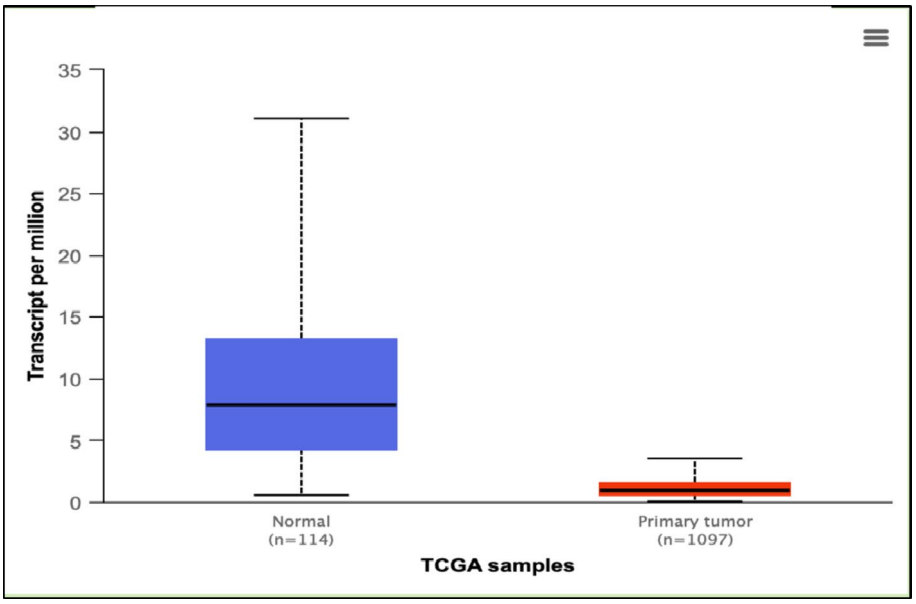

Fig. 9. The expression levels of the ANGPT1 gene using UALCAN website, Provide easy access to publicly available databases from the cancer genome atlas (TCGA).

region of differentially expressed genes (DEGs) in acute myeloid leukemia (LAML) was found to be correlated with their overexpression. Increasing methylation within the promoter region has the potential to modulate gene expression. Moreover, the phenomenon of hypermethylation leads to promoting oncogenesis by inversing well-established tumor suppressor genes expression[23,24].

MCF7 and MCF10A cell lines were used to validate our previous findings using qPCR technique[25]. The high expression level of *ANGPT1* was noticed in the MCF7 BC cell line compared to the MCF10A normal breast cell line. Similarly, Hayes and his colleagues found that *ANGPT1* was overexpressed in a human MCF7 BC cell line[25]. After treating both cell lines with 5-aza as a demethylating agent, the *ANGPT1* expression levels were strongly overexpressed compared to untreated cell lines. However, the MCF7 showed higher responses to the 5-aza than MCF10A. Treatment with 5-aza has the potential to induce reactivation of cancer-related genes in MCF-7 cells. This finding broadly supports the work of other studies in this area linking 5-aza-deoxycytidine with expression level. At low dosages, 5-aza functions as a demethylating agent, whereas greater doses of this medication induce

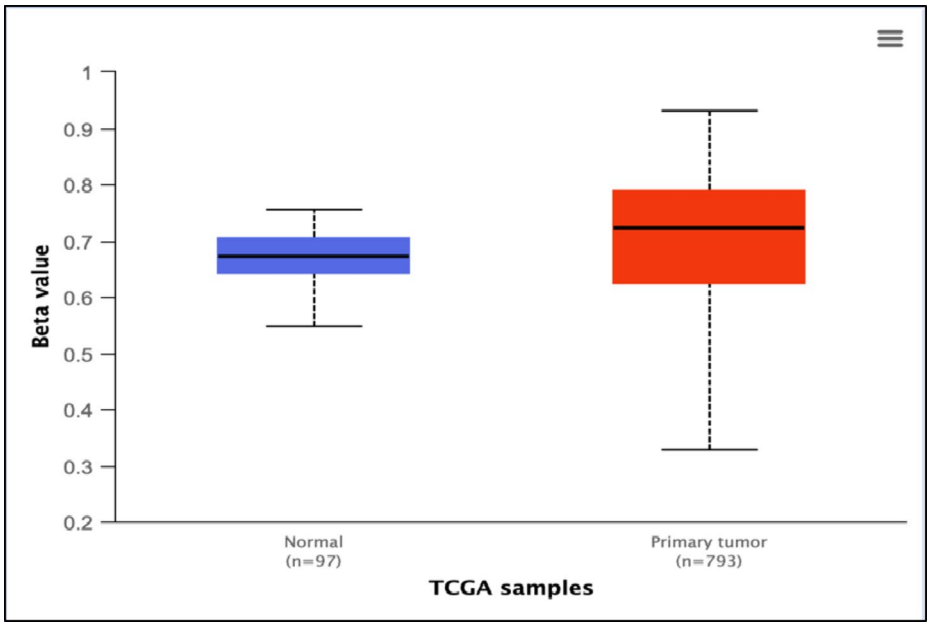

Fig. 10. The methylation level of ANGPT1 using the UALCAN page. Provide easy access to publicly available databases from the cancer genome atlas (TCGA). The beta value indicates the level of DNA methylation, ranging from 0 (unmethylated) to 1 (fully methylated).

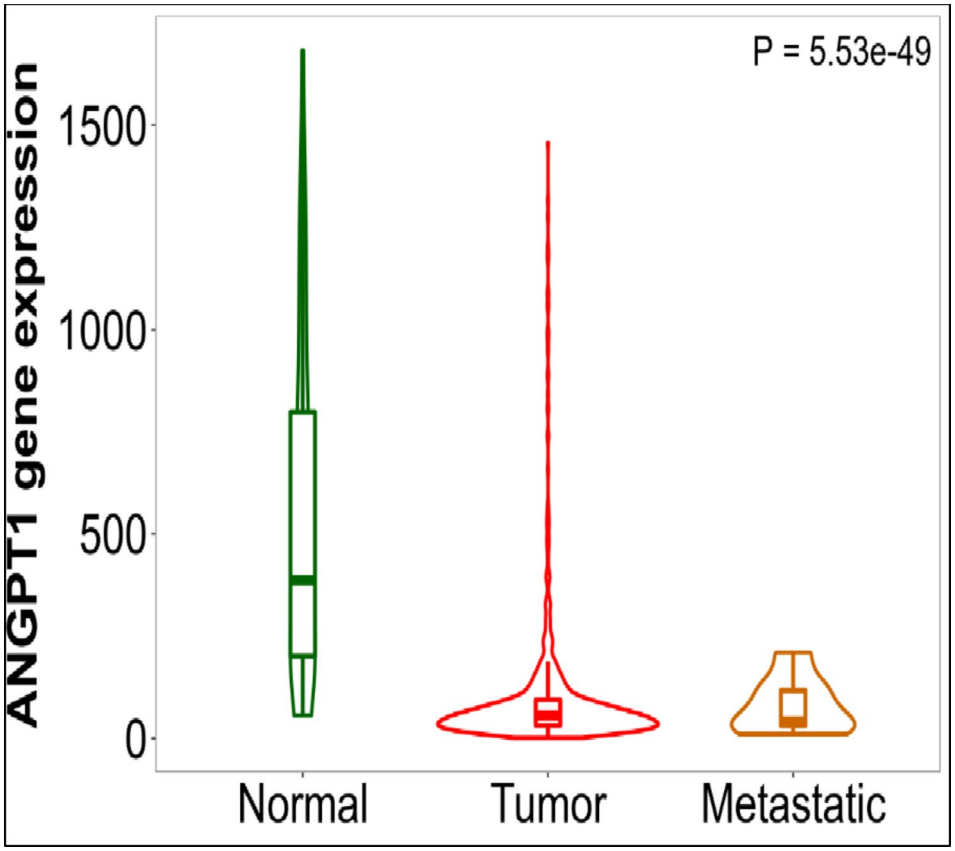

Fig. 11. The expression levels of the ANGPT1 gene using TNMplot page. The normal, tumor, and metastatic analysis page provides detailed analysis for a ANGPT1 gene in a breast invasive carcenoma tissue type using RNA-Seq based data.

Factors	BCSS			
	Hazard ratio	95% CI		*p* value
		Low *N* (%)	High *N* (%)	
ANGPT1 expression	1.389	1.012	1.905	**0.042**
Tumour_Grade	1.149	0.941	1.404	0.174
ER.Expr	0.899	0.674	1.199	0.470
Her2.Expr	1.601	1.218	2.103	< 0.001
PR.Expr	0.700	0.543	0.904	0.006
AGE	0.953	0.743	1.222	0.703
Tumor size	1.440	1.112	1.865	0.006
Lymphovascular invasion	1.364	1.086	1.713	0.008
Lymph node Stage	1.762	1.524	2.038	< 0.001

Table 4. Multivariate Cox proportional hazard regression analysis for predictors of BCSS in the BC METQBRIC cohort. Significant values are in bold.

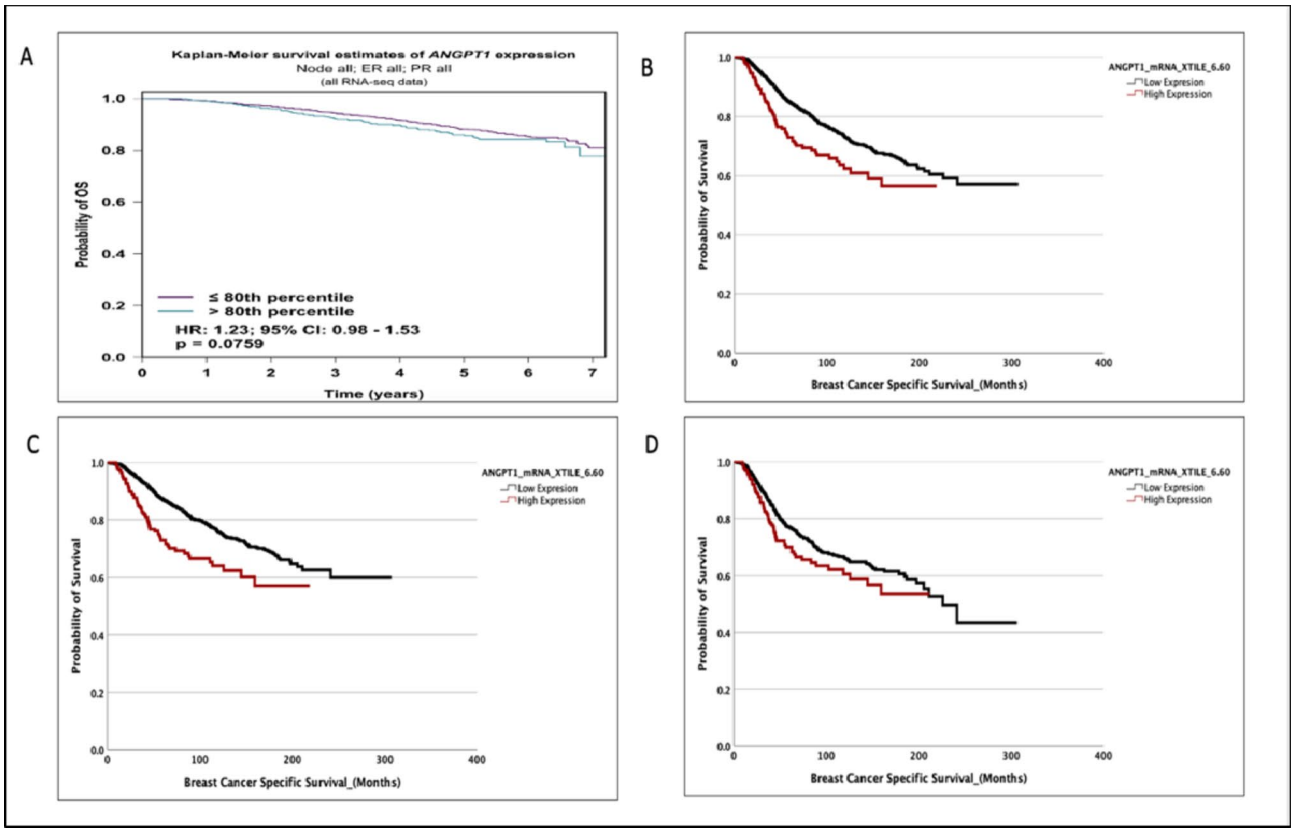

Fig. 12. Patients' breast cancer survival outcomes stratified by ANGPT1 expression. (**A**) Kaplan–Meier plots ANGPT1 gene expression. (**B**) The METABRIC cohort ANGPT1 expression. (**C**) Her2. Expression. (**D**) PR. Expression.

cell death because of cytotoxicity[26]. It suggested that DNA demethylation, leads to the re-expression of genes silenced in BC. The compound 5-aza has been seen to cause temporary cessation of the cell cycle and functions as a direct suppressor of methyltransferase activity, resulting in a reduction of methylation in newly created DNA[27]. In their recent publication, Danrong Ye and his team discuss similar findings. In order to determine the impact of the demethylation agent 5-aza on the downregulation of the DPT gene in cell lines, an investigation was conducted. It was shown that the 5-aza resulted in a substantial increase in both DPT mRNA and protein levels in BC cells[28].

In this study, several bioinformatics methods to expand our understanding of the possible functions of *ANGPT1* genes in BC. High *ANGPT1* expression was detected in patients with high histological grades,

negative estrogen and progesterone receptor status, and triple-negative breast cancer. Furthermore, *ANGPT1* was an independent prognostic marker. High expression of *ANGPT1* mRNA shorter BCSS. Our results were in concordance with study showing same prognostic effect of *ANGPT1* expression in Acute myeloid leukemia and BC[29].

The increased expression of *ANGPT1* may potentially lead to enhanced angiogenesis, by the development of novel blood vessels. In the context of cancer, angiogenesis plays a crucial role in facilitating tumor development by supplying essential nutrients and oxygen, promoting accelerated tumor proliferation and the potential for metastatic spread. Another finding was also reported by Nong B. et al. (2021) that high expression levels of *ANGPT1* had poor survival in Endometrial carcinoma patients[9]. On the other hand, previous studies have suggested that there is a significant association between high *ANGPT1* expression and a positive nodal stage ($P = .041$).

The CpG islands in our result detected in the first exon included a minimum CpG island size of greater than 100, and a CG percent greater than 50.0. The importance of this region is a significant regulatory locus with potential implications for cellular function, differentiation, or disease. According to gene card (www.genecards.org), *ANGTP1* has an Enhancer (Enhancer ID: GH08J107494) and 41 transcription factor binding sites such as *CTCF*, *ZNF600*, *REST*, *ZNF654*, and *RAD21*, the findings suggest that the *ANGPT1* probe did not detect methylation, it could mean that the specific site is not methylated. However, this does not rule out methylation at other CpG sites within the broader genomic region. As showed prior results hypomethylation in BC samples when included in a more extensive range 10x of the genome. In addition, when examined the impact of methylation level on *ANGPT1* expression by 5aza showed elevated in *ANGPT1* after treatment indicating that methylation plays a significant role and is a possibility that the *ANGPT1* probe inside the region could miss other methylation sites that might possibly exist in other areas of the gene or its surrounding regions. These results match those observed in an earlier study the CpG site *ANGPT1* did not achieve statistical significance at the epigenome-wide level in the context of delayed cerebral ischemia (DCI)[30].

The high expression of *ANGPT1* may serve as an indication of its involvement in the tumorigenic process and maybe associated with its roles in angiogenesis, which is a crucial factor in metastases. The elevated expression of this gene may also provide a disease prognosis and the potential for targeted therapeutics. No familial lineage suggests that epigenetic changes may play a more significant role in the development and advancement of IDC. The elevated expression of *ANGPT1* might potentially serve as a molecular indicator of these impacts. Additionally, the same data was reported by Ribeiro and his colleagues, who demonstrated a significant expression of *ANGPT1* in prostate cancer patients who were obese[31].

Materials and methods

Study population

This study aimed to examine the changes in gene expression and methylation levels in female breast cancer patients from Saudi Arabia. The study design is illustrated in (Fig. 13). In addition, Ethical approval for this study was obtained from the ethics committee of the biomedical ethics unit (No. 349 – 21) at King Abdulaziz

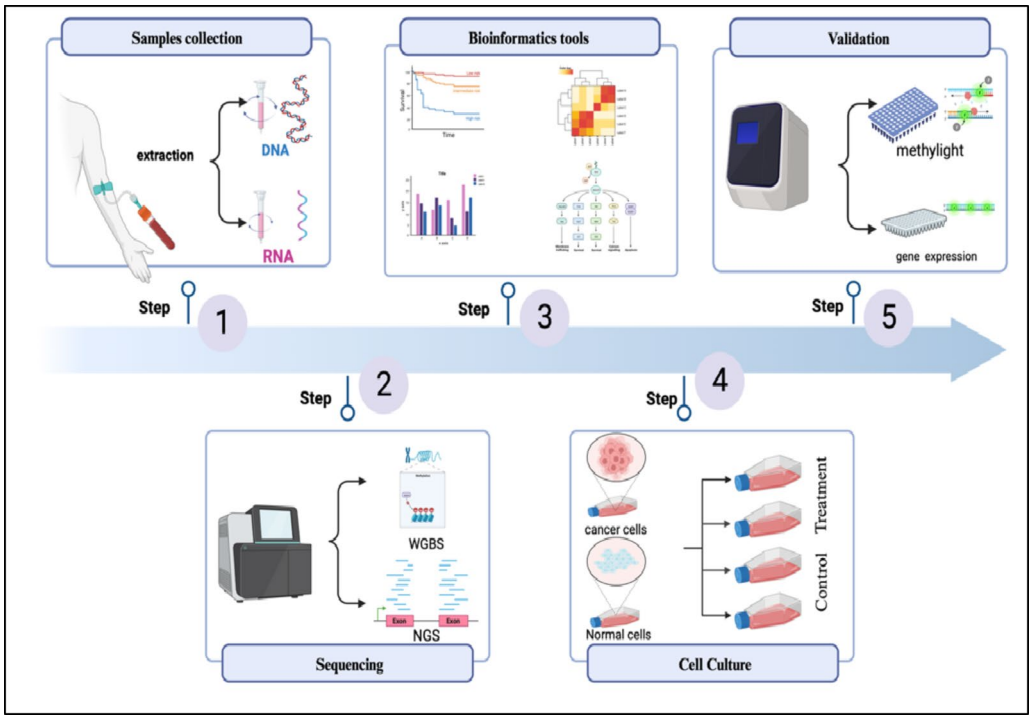

Fig. 13. The study flowchart.

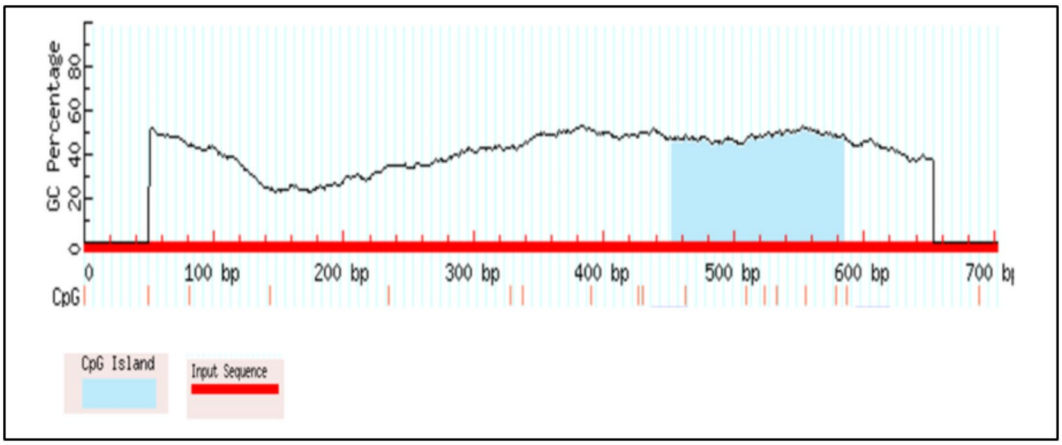

Fig. 14. The CpG island in first exon of ANGPT1. The criteria were used (CpG Island size > 100, GC Percent > 50.0, Obs/Exp > 0.6).

University in Jeddah. The samples used in this study consisted of 49 Saudi females, 21 of them were classified as "normal" and 28 were classified as BC patients from both King Abdulaziz University Hospital and the Medical Reference Clinics in Jeddah. Blood samples were obtained and preserved at a temperature of −80 °C using 4 mL tubes containing EDTA anticoagulant.

DNA extraction

The genomic DNA was extracted from whole blood that had been maintained using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), following the instructions provided by the manufacturer[32]. The NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific Inc., USA) was utilized to assess the quantity and quality of DNA by determining the absorbance ratio at 260 nm and 280 nm. DNA samples displaying a ratio of 1.8 were regarded as adequate.

Human whole genome bisulfite sequencing (WGBS)

WGBS was used to analyze the DNA methylation patterns across the entire genome of an individual. 100ng/uL of DNA from each sample was submitted to Novogene (Hk) Company Limited for WGBS next-generation sequencing technology, which is a comprehensive and impartial way to examine DNA methylation across the genome at the individual nucleotide level. This technique involves treating DNA with sodium bisulfite prior to performing high-throughput DNA sequencing. The Novaseq 6000 platform was employed for data generation following the manufacturer's instructions to achieve a genome coverage of 10x.

DNA bisulfite conversion

The EpiTect Bisulfite Kit (Qiagen, Germany) was employed for all samples. The purpose of converting cytosines that were not methylated in the DNA strand into uracil (U). The bisulfite solution was made according to the manufacturer's instructions[33].

MethyLight qPCR

To ascertain the ratio of methylated CpG dinucleotides, a methylight assay is employed. Adhering to the manufacturer's instructions outlined in the EpiTect MethyLight PCR Kit (Qiagen, Germany)[34]. The MethyLight test utilizes a methylation-specific TaqMan probe. For 49 samples. In this study, the primers were designed and selected in proximity to the anticipated CpG islands using the MethPrimer software. These primers were specifically targeted towards the first exon of *ANGPT1*, spanning a length of 700 base pairs (from position 452 to position 610). The criteria employed in this study included a minimum CpG island size of greater than 100, a GC percent greater than 50.0, and an observed-to-expected ratio greater than 0.6. As shown in (Fig. 14). A TaqMan probe with a 5' FAM reporter dye was used to detect unconverted cytosine in the target gene. Additionally, another TaqMan probe with HEX dye is utilized to evaluate unconverted cytosine in the reference gene (collagen). A 10X primer-probe combination was prepared for each ANGPT1, which includes a 5' reporter dye (FAM). *ANGPT1* gene primer F 5`ATTTAATATGGGTAATGTGTTTA3`/ R 5` TATAAAACATCTCTCTACAAAA3` Probe 5`FAM TTAGAATACGATGGTAATTGTCGT BHQ13`. Tm ℃ 47 for primers and 54.6 for prob. In addition, COLLAGEN, which includes a 5' reporter dye (HEX) F 5'TCTAACAATTATAAACTCCAACCACCAA3'/ R5`5'GGGAAGATGGGATAGAAGGGAATAT3'. Probe 5`HEX CCTTCATTCTAACCCAATACCTATCC CACCTCTAAA BHQ13`. Tm = 54.8, 55.6, 73.2 °C respectively. Three controls were employed in methylight quantitative polymerase chain reaction (qPCR) EpiTect Control DNA Set (Qiagen, Germany) used to assess the precision and selectivity of the primers and probes. The experimental setup included three control samples: a positive control consisting of 100% methylated converted DNA, a negative control consisting of non-methylated converted DNA, and another negative control consisting of unmethylated unconverted DNA. The stander and melt were utilized for the TaqMan probe, employing a 1:5 serial dilution.

RNA extraction
The genomic RNA was extracted from whole blood samples following the protocol provided by the manufacturer, utilizing an RNA isolation kit specifically designed for blood samples (Haven, Jeddah, Saudi Arabia)[35]. The NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific Inc., USA) was utilized to assess the quantity and quality RNA by determining the absorbance ratio at 260 and 280 nm. RNA samples exhibiting a ratio of 2.0 were deemed suitable.

cDNA synthesis
The RNA samples obtained from blood were subjected to cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany)[36].

RNAseq analysis
Whole RNA sequencing was done by using RNA sequencing via Illumina platforms, based on the mechanism of SBS (sequencing by synthesis). (100ng/uL) RNA-extracted samples were sent to NOVOGENE (HK) COMPANY LIMITED. According to the manufacturer's instructions[37].

qPCR analysis
Gene expression differences between breast cancer and healthy control samples were estimated using three technical replicates for each sample of 24 biological replicate samples. The BIO RAD CFX96 Real-Time System (BIO-RAD CA, USA) technology was employed to determine the expression level of target genes. The experiment employed the EverGreen Universal qPCR Master Mix, which was produced by researchers at (Haven, KSA). Using Haven's custom assay for GAPDH (Cat. No. PCR-PDA-HSA-GAPDH-11 Haven, Jeddah, KSA) as a reference gene. The *ANGPT1* gene primer was F 5`CCTGATCTTACACGGTGCTG3`/ R 5`CAAACCACCATCCTCCTGTT3`. TM = 60 °C. Five µl EverGreen universal qPCR master mix (2X) was added, 1 µl of primer was added, 100 ng of cDNA, and 3 µl of nuclease-free water to reach 10 µl maximum reaction volumes. Then, qPCR was programmed as follows: initial denaturation at 95°C for 5 min, denaturation at 95° C for 15 s, and annealing temperature at 54–61 °C for 60 s with 45 cycles.

Cell culture analysis
Human BC cell line MCF 7 were obtained from the immunity unit in King Fahad Research Center were cultivated and Human normal breast cell line MCF10A was acquired from Health Science Research Center at Princess Nourah bint Abdulrahman University. MCF7 and MCF10A were cultured in DMEM (Dulbecco's Modified Eagle Medium). 10% FBS (Fetal Bovine Serum) and 1% P/S (Penicillin/Streptomycin) were routinely added to both media. When cells attained approximately 80% confluence, they were passaged 1:2 in fresh medium in a 37 °C humidified incubator containing 5% CO2.

Demethylation by 5-aza-2`deoxycytidine
The cells were treated with a 5 µM dose of 5-aza-2`deoxycytidine. The medium was supplemented with 5-aza and subjected to incubation for a period of 7 days. Subsequently, all cell types were standardized to a cell count of 105. The cellular components were harvested on the third and seventh days. Following this, RNA was extracted with the intention of conducting gene expression analyses. The average of triplicate Ct values for each sample was determined using automated machine thresholds, and the relative expression level of *ANGPT1* was computed using the Pfaffl equation in relation to the housekeeping gene GAPDH. The Pffafl equation was used to ascertain the comparative quantity of RNA pertaining to the target gene across many samples.

Bioinformatics analysis
In order to conduct a more comprehensive investigation into the predictive significance of the previously mentioned Differentially Expressed Genes (DEGs) in BC, we acquired gene expression data from two datasets: TNM-plot (https://www.tnmplot.com/) and UALCAN (http://ualcan.path.uab.edu/index.html). These datasets collectively encompass 1097 primary tumors, 7 metastatic tumors, and 114 normal tissue samples. The prognostic and molecular relevance of the identified differentially expressed genes (DEGs) in BC were validated using the online analytical module Kaplan Meier Plotter ($n = 3951$) and the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset ($n = 1980$) and (TCGA) dataset ($n = 854$).

Statistical analysis
The statistical analysis for this research was conducted using the IBM SPSS® Statistics software program (IBM Company, New York, NY, USA), specifically with IBM SPSS Statistics, version 26. A p-value less than 0.05 was deemed to be statistically significant. The association between the clinical-pathological features and *ANGPT1* expression was assessed using the Chi-square test. The assessment of the prognostic significance of *ANGPT1* expression was conducted using Kaplan-Meier survival curves. The Cox proportional hazard method was used to evaluate the multivariate survival analysis BCSS (Breast Cancer Specific Survival). The statistical significance of the clinical-pathological factors and survival was assessed by using a p-value below 0.05. To analyze qPCR data the Excel tool used student t-test was performed for each group of BC patients and normal a p-value below 0.05.

Conclusions
In the context of BC, our investigation has generated substantial data indicating a strong correlation between elevated levels of *ANGPT1* expression and its hypomethylation status speculating its important role in the oncogenesis and progression of BC. Interestingly, the presence of abnormal gene expression and methylation

patterns was shown to be strongly correlated with worse clinical outcomes in individuals diagnosed with BC. The possible epigenetic marker, *ANGPT1*, may undergo hypomethylation, leading to increased expression and possibly playing a role in tumor growth and aggressiveness, and may function as an oncogene. The aforementioned results highlight the potential of *ANGPT1* as a promising predictive biomarker in BC and indicate the need for more investigation to clarify the exact molecular processes behind this correlation. Furthermore, the exploration of the *ANGPT1* pathway as a potential therapeutic target might provide a new and innovative approach for the treatment of BC in individuals who have elevated *ANGPT1* expression levels.

Data availability

The transcriptomic and associated clinical data utilized in this study is publicly available from the cBioPortal for Cancer Genomics , KM-Plotter , bc-GenExMiner v4.7 database40 and TNM-plot (https://www.tnmplot.com/) and UALCAN (http://ualcan.path.uab.edu/index.html). The RNAseq data reported here was derived from pat ient specimens (blood samples) and as such is identifiable. Because of this the raw sequencing reads and BAM files cannot be made publicly available via NCBI-GEO nor any other public domain data. The data will be made available on request and approval of the study data management committee and in compliance with the requirements of the Ministry of Health in Kingdom of Saudi Arabia.

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Author contributions
Conceptualization, M.A.A., S.S., S.H. and S.A.; methodology, M.A.A., S.S., S.A.A., S.H. and S.A., software, M.A.A., and S.S; validation, M.A.A., S.S., S.A.A and S.A; formal analysis, M.A.A. and S.S; investigation, A.L., S.K., H.A., A.E. and S.A.A., resources, M.A.A. A.L., S.K., S.S., N.H., S.A.A., K.A., R.F. and H.A.; data curation, M.A.A., S.S. and S.A; writing original draft preparation, M.A.A., S.S., S.A., and S, H.; writing review and editing, M.A.A., S.S. H.A., S.H. and S.A.; visualization, N.A., M.G., N.S., G.A., A.E. and H.A ; supervision, S.A and S.H. project administration, S.A.; funding acquisition, H.A. All authors read and approved the final manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Institutional review board statement
The investigation was conducted in accordance with the declaration of Helsinki and was approved by the King Abdulaziz University biomedical ethics unit's ethics committee (No.349 – 21). The participants signed informed consent forms.

Informed consent statement
Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patients to publish this paper.

Additional information
Correspondence and requests for materials should be addressed to S.A.

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