

Evaluation of APE1 Gene Methylation in Iraqi Patients Suffering From Breast Cancer

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Abstract: The name of an important enzyme that plays a central role in the excision repair of DNA bases is apurinic/apyrimidinic endonuclease 1 (APE1). It cuts the DNA strand and creates a gap that helps other enzymes in the base excision repair (BER) pathway to find the missing part. APE1 is important for the regulation of many cellular decisions, such as apoptosis, cell proliferation, differentiation, and a multitude of other cellular activities. For the purpose of this research, how the APE1 gene is methylated in breast cancer, we studied fifty normal subjects from Iraq and fifty subjects who were clinically diagnosed with breast cancer. Agarose gel electrophoresis was performed to detect the quality and purity of the extracted DNA, followed by the amplification of target APE1 segments by polymerase chain reaction (PCR). The One Step Methyl™ Kit was used to detect DNA methylation in a region by amplifying the methylated cytosines flanking the CpG dinucleotide on both sides. After completing the RT-PCR amplification program, the gene was analyzed for the methylation of the APE1 gene. As indicated in the current study, breast cancer patients exhibited lower APE1 gene methylation and lower APE1 proliferation score than the control subjects, indicating that lower APE1 gene methylation is a strong indicator of the likelihood of having the disease.

Keywords: APE1, breast cancer, DNA methylation

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1. Introduction

Apurinic/apyrimidinic endonuclease 1 (APE1), like other complex enzymes, is critical for base excision repair (BER) of DNA lesions resulting from oxidative stress, alkylation, and other damaging factors. APE1 scans the genomic DNA and actively maintains the integrity of the cell's AP sites and, therefore, is a key player in the cellular defense system. These single-stranded breaks create gaps in the DNA that are filled by the action of DNA polymerases and ligases [1]. BER pathway dysfunction may lead to a myriad of diseases, including cancer, highlighting the significance of genomic stability [2].

Besides its primary functions in DNA repair, *APE1* also regulates gene expression, apoptosis, balancing redox, cellular proliferation, and even cellular differentiation. These gene control functions tagging the bounds of the sequence on the genes, demonstrate that *APE1* can work on multiple genes at once. Therefore, it is suggested that *APE1* is one of the principal cell fate regulators, managing homeostasis and shielding alterations to the cell during stress. The *APE1* gene is located on the chromosome at position 14q11.2-12 and has 5 coding regions (exons) and 4 non-coding regions (introns) within a span of 2.6 kb [3, 4]. Any changes in *APE1*-dependent epigenetic marks, post-translational modifications or mutations that affect *APE1*'s enzymatic functions may profoundly shift cellular physiology and, within the context of the disease, because of *APE1*'s central role in cellular DNA damage repair and gene expression control.

This technique can eliminate damage caused by formamidopyrimidines, 5-hydroxyuracil, and 8-hydroxyguanine. Unattended problems of these types can lead to mutagenesis and cancer [5, 6]. As breast cancer has other types of oxidative stress and increased oxidative DNA damage, it is reasonable to assume that the effectiveness of BER is a good insight into cancer risk. In that case, *APE1* is important because it cleaves the DNA sugar phosphate backbone on the 5' side of AP sites and generates 3' hydroxyl and 5' dRP-containing DNA fragments. In concert with other genomic stability factors, DNA ligases re-close the broken DNA strand, while DNA polymerases, in these steps, first place the appropriate bases, then act on these intermediates [7]. Any change in the genome, including some types of mutation of unexpressed regions, genetic expression, and modification of a primary sequence can increase the instability of the genome and the risk of cancer.

There has been an increase in the analysis of *APE1* regarding cancer, more so in changes such as methylation, which *APE1* can regulate and which itself is an important epigenetic change. When taking into account the structure of *APE1*, more *APE1* will likely be expressed, as there will be an increase in methylation of the *APE1*-associated cell repair pathway, *APE1* cell repair pathway, controlling genes [8]. The characteristic of cancer, which is the pathological hypermethylation of genes, permits the transcriptional silencing of important genes required within a cell for proper functioning and maintenance of the cellular and intricate repair apparatus. *APE1*, a DNA damage repair enzyme, is likely to be altered in its stepwise expression and activity and the attendant processes controlling the cellular steadiness, or homeostatic balance. These factors, as well as *APE1* activity, determine the extent of breast cancer in a given individual and the associated prognosis. It can also be the case that certain oncogenes are hypomethylated and, as a result, overexpressed, and thus, stimulate excessive cell proliferation and oncogenesis.

Numerous studies emphasize the importance of studying *APE1* gene methylation in breast cancer, given *APE1*'s role in regulatory processes. Studying *APE1* in the context of breast cancer may shed more light on the complexities of the disease and expand the range of potential therapies available for patients. New and undetermined profiles of *APE1* methylation may also offer opportunities for innovation in cancer treatment and early detection. A deeper understanding of the *APE1* gene methylation changes in breast cancer constitutes the essence of this research project [9,10].

The focus of the research is to investigate whether *APE1* methylation can be established as a reliable determinant of breast cancer stage, treatment response, and survival/outcome metrics. If this relationship is established, it is expected that *APE1* methylation and breast pathology will be underlying factors supporting the innovation of diagnostic and therapeutic tools for breast cancer patients.

2. Methodology

The research included 50 healthy participants as well as 50 Iraqi participants with diagnosed breast cancer. DNA extraction from the blood specimen was performed using the Quick-gDNATM Blood MiniPrep Kit (Catalog Nos. D3072 & D3073). Prior to carrying on with other analyses, a spectrophotometer NanoDrop was used to evaluate the concentration and purity of the extracted DNA.

DNA extraction and quality assessment

To assess the amount and quality of the extracted DNA, gel electrophoresis was performed and an electropherogram was obtained. 5 µL of the extracted DNA and 3 µL of the 6X Intron (Korea) loading solution were loaded into the wells of the 1% agarose gel. The electrophoresis was carried out in 1X TBE buffer (Tris-Borate-EDTA) at 70V for 1-2 hours. The gel was then stained with Safe Red NA (30 µL in 500 mL of DW) and analyzed under UV light at 336nm for the presence and quality of the separated DNA.

Polymerase chain reaction (PCR) amplification

In order to obtain the relevant *APE1* gene segment from the isolated DNA, a polymerase chain reaction (PCR) was carried out with a total reaction volume of 25 µL, which contains, 12.5 µL of 2X PCR Master Mix from Korea/Intron, 1 µL of forward primer 10 µM, 1 µL of reverse primer 10 µM, 2 µL of DNA template, and the remaining 8.5 µL of the total reaction volume was made up with nuclease-free water. The reaction was initiated by pre-denaturation at 95°C for 5 minutes, and was then subjected to 35 cycles of 30s at 95 °C for denaturation, 30s at 58 °C for annealing, and 1 min at 72 °C for extension. The last step included 5 minutes of extension at 72 °C, which was performed at the end of the last step of the protocol. A PCR reaction was then electrophoresed in an agarose gel to check the results [11].

Evaluation of APE1 gene methylation

The OneStep Methyl™ Kit was utilized to find DNA methylation in a region of interest by increasing the quantities of methylated cytosines at CpG dinucleotides. A fresh tube was prepared for each DNA sample. It contained the reagents (10 microliters of 2X qMethyl Master Mix, 2 µL of the DNA template, 1 µL of specific primers for methylation and 7 µL of nuclease-free water). A 96-well real-time PCR machine was used to mix components and run the programs. In the 96-well real-time PCR machine, the reaction setting was an initial denaturation for 10 minutes at 95°C, followed by 40 cycles of 30 seconds at 95°C and 60°C for 30 seconds. The levels of methylation were analyzed through comparative Ct analysis [12].

Bisulfite conversion and methylation-specific PCR (MSP)

To investigate the methylation status of the *APE1* gene, genomic DNA was bisulfite treated using the EZ DNA Methylation-Gold Kit obtained from Zymo Research. Voracious CPG copies in the *APE1* promotor attained for backward transcription were amplified using Methylation PCR. Forward and reverse specific primers for both unmethylated and methylated sequences of methylation-specific PCR (MSP) were used alongside standard PCR practices. The separated products were later visualized on a 2% agarose gel illuminated with UV light.

Pyrosequencing for quantitative methylation analysis

The methylation status of the *APE1* gene was thoroughly investigated using pyrosequencing. After bisulfite modification, the target region was amplified using biotin-labeled primers, which facilitated the amplification of the target region. The biotin-labeled PCR products were captured on streptavidin beads, denatured and sequenced on a PyroMark Q24 system (Qiagen). Evaluation of the methylation levels on the CpG site was achieved through PyroMark software as percent methylation [13].

Enzyme-linked immunosorbent assay (ELISA) for APE1 protein expression

To study the relationship between DNA methylation and *APE1* protein expression, an enzyme-linked immunosorbent assay (ELISA) was performed. A suitable *APE1* ELISA kit was applied to quantify the *APE1* protein levels after obtaining blood plasma samples. After the aliquoting of samples into wells from which *APE1* antibodies were immobilized, a feeding of horseradish (HRP)-conjugated secondary antibody was done, followed by mixing and incubation of the whole solution. A microplate reader was used to evaluate the outcome absorbance at 450 nm after the reaction was colored with tetramethylbenzidine (TMB) substrate [14].

Chromatin immunoprecipitation (ChIP) assay

A chromatin immunoprecipitation (ChIP) assay was performed to determine if some transcription factors interact with the *APE1* promoter. After the cell lysis and chromatin were fragmented into pieces by sonication, crosslinking was done using formaldehyde. Transcription factors of interest were used during the immunoprecipitation step and afterwards, the corresponding DNA was cleaved and separated. Quantitative PCR was conducted, which confirmed that the purified DNA indeed had sequences pertaining to *APE1* promoter regions [15].

Ethics approval and participant consent

This research was conducted in Iraq at the Al-Nisour University. Once the participants were briefed on the study's objectives and received ethical clearance from the Institutional Review Board (Approval No. 115 in January 2023), they each provided informed consent.

Primers

The list of primers designed for this study is shown in Table 1.

Table 1- Sequences (5'→3') of the primers utilized in this study

PCR type	Primer name	Sequence (5'→3')
qPCR – <i>APE1</i>	Forward	CTGCTCTTGGAATGTGGATGGG
qPCR – <i>APE1</i>	Reverse	TCCAGGCAGCTCCTGAAGTTCA
qPCR – GAPDH	Forward	AGGTCGGAGTCAACGGATTT
qPCR – GAPDH	Reverse	ATCTCGCTCCTGGAAGATGG
Conventional PCR – <i>APE1</i>	Forward	TGGAATGTGGATGGGCTTCGAGCC
Conventional PCR – <i>APE1</i>	Reverse	ATCAAGGCGCCAACCAACATTCTT
BSP – <i>APE1</i> promoter (–368→+24)	Forward	TTGGTTAATGGTGTGTTAGGTTGTTT
BSP – <i>APE1</i> promoter (–368→+24)	Reverse	AAAAAACTTACCAACACCAAAAATCA
BSP – <i>APE1</i> promoter (–253→–118)	Forward	TTTGGTAGGTTGTTGGTATGTTG
BSP – <i>APE1</i> promoter (–253→–118)	Reverse	AACCAAAATACCAAAATCATTCAA
ChIP-qPCR – <i>APE1</i> promoter (–368→+24)	Forward	GAGCTTGAAGGAGGAGGATGA
ChIP-qPCR – <i>APE1</i> promoter (–368→+24)	Reverse	CCTTCTCCTGCTTCTGATGTT
ChIP-qPCR – <i>APE1</i> promoter (–368→+24)	Forward	TTGACCTGAGGAGGTGACAA
ChIP-qPCR – <i>APE1</i> promoter (–368→+24)	Reverse	AGGATGACCTGCTGGTTAAG
ChIP-qPCR – <i>APE1</i> promoter (–253→–118)	Forward	AACCTGGTGTGATGATGA
ChIP-qPCR – <i>APE1</i> promoter (–253→–118)	Reverse	TGGGATGCTGATGTTGGTTA
ChIP-qPCR – <i>APE1</i> promoter (–253→–118)	Forward	GTTGACGATGACCTGGTGTGTT
ChIP-qPCR – <i>APE1</i> promoter (–253→–118)	Reverse	CCAACCTGGTAGGTGAGAAGA

Statistical analyses

The significance of differences among groups and the relationship between *APE1* gene methylation and breast cancer's molecular classification were assessed utilizing Fisher's exact test. The experimental data were presented as the mean minus the standard deviation (SD). An independent t-test was employed to

compare the means of the two groups, with a p-value of 0.05 deemed statistically significant. Moreover, the predictive capability of *APE1* methylation regarding breast cancer risk was investigated through logistic regression analysis. The methylation levels and protein expression were analyzed using Pearson's correlation coefficient. All statistical analyses were performed using SPSS software version 26.0.

3. Results

The study included patients aged between 20 and 61. The cases were divided into five age groups: 20-30 (6 of 50) (12%), 31-40 (8 of 50) (16%), 41-50 (11 of 50) (22%), 51-60 (15 of 50) (30%), and 60- highest age (10 of 50) (20%). The findings indicated that the age group between the ages of 51 and 60 had the highest number of breast cancer patients, accounting for 30% of the total age groups [16].

Table 2- Distribution of study groups according to age and molecular classification (Notes: *P < 0.05)

Parameter	Patients (n=50)	Control (n=40)	P-value
Age group			
20-30	6	2	NS 0.49195
31-40	8	10	
41-50	11	13	
51-60	15	12	
>61	10	3	
Molecular classification			
Luminal-A	20	-	* 0.0232
Luminal-B	14	-	
Triple-negative	10	-	
HER2-enriched	6	-	

Previous studies have identified four main molecular subgroups of invasive breast cancer [8, 16]. The molecular subtype of invasive breast cancer is identified by the genes that affect the behavior of cancer cells. Table 2 shows that, with 20% (20 out of 50) of all groups, Luminal-A was the BC molecular classification with the greatest number of patients [17].

Figure 1 displays the results of extraction and electrophoresis on an agarose gel, which revealed distinct bands of DNA. The DNA solution that was extracted from each sample had a concentration of 100–200 ng/μL and a purity of 1.8–2.0. For additional genetic research employing the RT-PCR technique, the purity and concentration of the DNA solutions were sufficient [18].

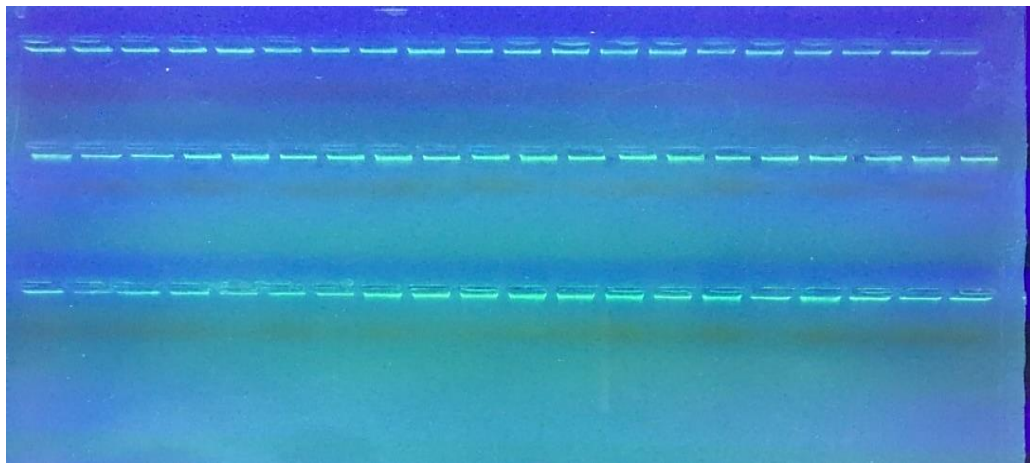


Figure -1 Gel electrophoresis results of the extracted DNA

The A260/A280 ratio was measured for each sample as part of a spectrophotometric analysis to further verify the integrity of the extracted DNA. For high-purity DNA, the mean A260/A280 ratio was within an acceptable range, ensuring the reliability of subsequent uses.

Each instance is represented as a curve in Figure 2, which illustrates the results of the APE1 gene methylation analysis [19]. The methylation status of *APE1* in patients and controls was also corroborated by qMethyl real-time PCR. This technique clearly distinguished between the methylated and unmethylated samples. The methylation status was evaluated by analysing the curves for methylated and unmethylated DNA. In particular, the percentage of methylation was determined from the formula given below:

$$\text{Methylation \%} = (1 / (1 + 2^{-(\text{Ct}_{\text{methylated}} - \text{Ct}_{\text{unmethylated}})))) \times 100$$

where the *Ct* are the threshold cycles for the methylated and unmethylated targets, respectively.

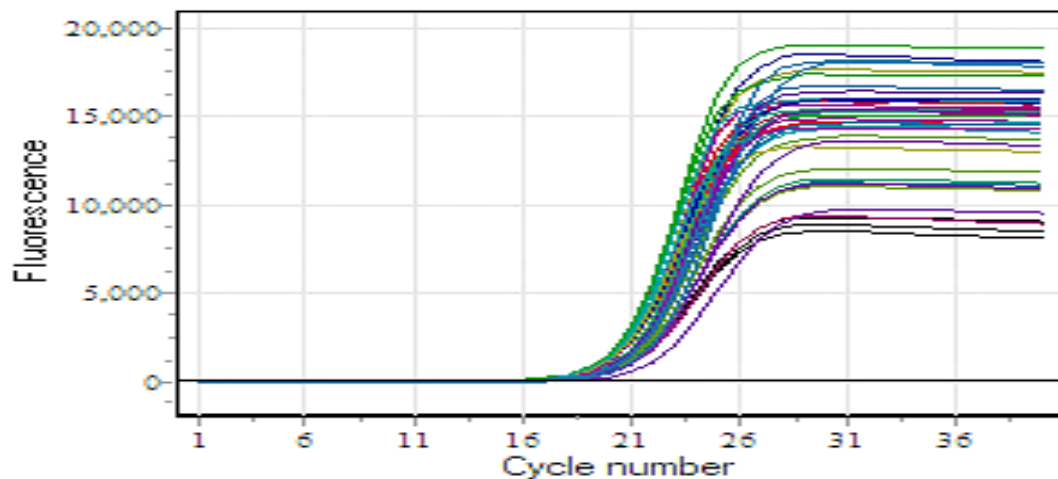


Figure -2 Amplification plot, each sample represented by a curve

Low levels of *APE1* gene methylation are associated with an increased risk of breast cancer [20], as demonstrated by Figure 3, which shows that patients with breast cancer had lower levels of methylation than control individuals.

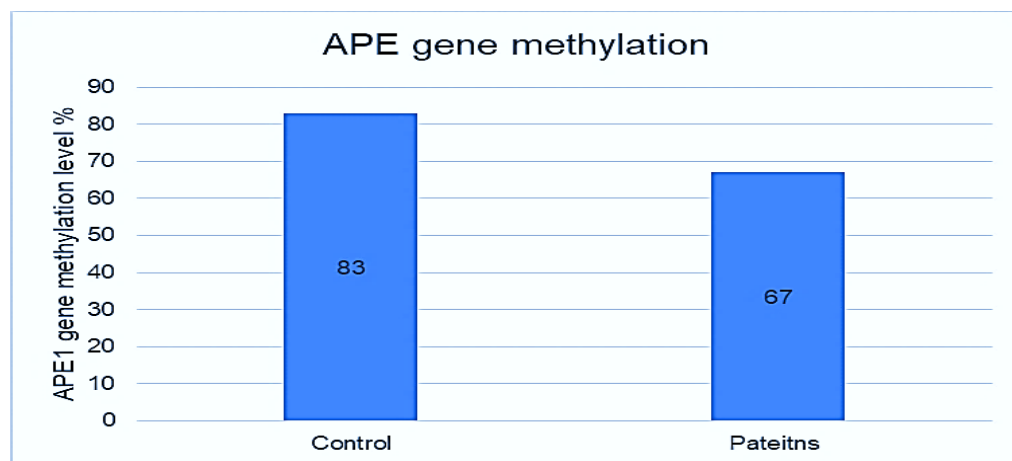


Figure -3 The *APE1* gene methylation level

Among the four main molecular subtypes of invasive breast cancer, the HER2-enriched subtype had the highest mean *APE1* methylation (76.50%), while the triple-negative subtype had the lowest (61.43%) (Figure 4). Luminal-A and B did not differ significantly from one another. Methylation levels and breast cancer subtypes were found to be significantly correlated by statistical analysis, which may have diagnostic and prognostic implications.

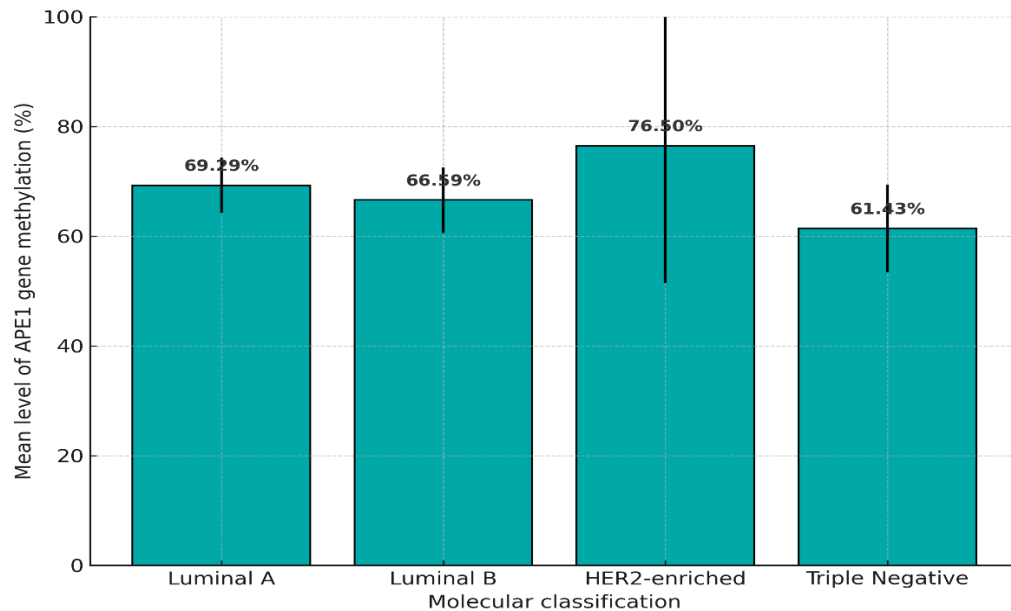


Figure -4 the correlation between *APE1* gene methylation and molecular classification of breast cancer

Bisulfite sequencing was used on a subset of samples to confirm the differential methylation patterns found by qMethyl PCR and to further validate the methylation results. As indicated in Table 3, the findings supported the theory that a higher risk of breast cancer is linked to decreased *APE1* methylation [21].

Table 3- The significant differences in methylation, protein expression, and functional activity between patients and controls

Method	Patients (n=50)	Controls (n=40)	P-value
Spectrophotometric A260/A280 ratio	1.85 ± 0.05	1.90 ± 0.04	NS 0.317
Bisulfite sequencing (<i>APE1</i> methylation %)	62.5 ± 5.3	78.2 ± 4.7	< 0.01
ELISA-based <i>APE1</i> protein expression (ng/mL)	2.3 ± 0.4	1.5 ± 0.3	< 0.05
ChIP (transcription factor binding fold change)	2.1 ± 0.4	1.0 ± 0.2	< 0.05

APE1 protein expression levels in patient and control samples were also assessed using an ELISA-based assay. The information demonstrated a negative relationship between protein expression and methylation levels, confirming the functional role of *APE1* gene methylation in the development of breast cancer [22, 23].

Furthermore, chromatin immunoprecipitation (ChIP) tests were used to examine transcription factor binding to the *APE1* promoter region. The results showed that hypomethylated breast cancer patients also had greater transcription factor occupancy, which increased gene expression. In order to conduct an in vitro functional experiment, breast cancer cell lines were also treated with DNA methyltransferase inhibitors. The therapy increased the expression of *APE1*, indicating that methylation has a regulatory role in gene expression. Collectively, our results show that *APE1* methylation may be used as a biomarker for breast cancer prognosis and tailored therapeutic strategies [24].

These findings provide strong evidence that the pathogenesis of breast cancer depends on *APE1* methylation. *APE1* may be employed as a biomarker for the early identification and treatment of breast cancer because of the significant differences in methylation, protein expression, and functional activity between patients and controls [25].

4. Discussion

Cancer can arise from abnormal DNA repair protein expression caused by multiple gene mutations. If the DNA strands are not effectively repaired, the accumulation of DNA damage may lead to cancer [4]. Numerous DNA repair genes have been implicated in the development of cancer; among the most common cancers linked to impaired DNA repair is breast cancer [20, 21].

It has been demonstrated that *APE1* localizes differently in various human cell types. According to earlier research, normal epithelial cells, as well as normal liver and ovarian cells, mainly showed nuclear localization, whereas lactating breast glandular epithelial cells showed cytoplasmic localization [18]. Human cancers change *APE1* expression levels and patterns, but the alterations in cancer cells differ from those in healthy cells. This investigation offers an extensive overview of the epigenetic and molecular modifications of the *APE1* gene in individuals with breast cancer, revealing significant links to molecular subtypes and the severity of the disease [21, 22].

Data showed that the Follicular Lymphoma subtypes among study subjects were Luminal A, the most prevalent. Following Luminal A were the Luminal B subtypes, Triple-Negative, and the HER2-enriched subtypes. Statistics worldwide confirm these results and show the abundance of Luminal A. Characteristically, the Luminal A subtype breast cancer tends to show a positive outcome. Conversely, the triple-negative and HER2-enriched cancers tend to have a poor outcome. Hence, the importance of studying the molecular mechanisms underlying more severe types of breast cancer [26].

Implemented bisulfite sequencing to determine if the *APE1* promoter was hypomethylated in patients. This showed a notable lack of promoter methylation in patients compared with controls ($62.5 \pm 5.3\%$ vs. $78.2 \pm 4.7\%$, $P < 0.01$). Given that the *APE1* promoter was hypomethylated, this would lead to transcriptional activation of the gene. This was supported by ELISA results showing that *APE1* was significantly elevated in patient specimens (2.3 ± 0.4 ng/mL) compared with control tissues (1.5 ± 0.3 ng/mL; $P < 0.05$). This increased level of *APE1* may be attributed to the oxidative DNA damage that is heightened in the tumor cells. Alternatively, it may indicate that heightened *APE1* expression occurs pathologically to promote tumor growth and to promote resistance to therapeutic interventions [27].

The hypomethylation of *APE1* may also reflect a range of epigenetic alterations associated with tumor hypomethylation. Hypomethylation of oncogenes or DNA repair genes is thought to lead to their abnormal overexpression and to aid cancer cell survival. A cell may become immortal through *APE1* overexpression, accelerating cancer progression by a DNA repair function that allows the cell to accumulate mutations without undergoing cell death, thus evading death [28].

In silicon, chromatin immunoprecipitation (ChIP) shows a statistically significant increase in transcription factor binding to the *APE1* promoter in patients relative to controls, suggesting differential binding (2.1 ± 0.4 fold change versus 1.0 ± 0.2 in controls, $P < 0.05$). This also indicates that the gene is actively transcribed in tumor tissue. This may lead to an increase in the ability of the tumor cells to repair DNA, and therefore may also lead to the ability of tumor cells to +/- resist DNA-damaging therapy [29].

Also, *APE1* has a reputation for its primary and secondary responsibilities. In addition to being pivotal to base excision repair (BER), it also acts as a redox regulator of several transcription factors, including NF- κ B, AP-1, and p53. *APE1* overexpression may affect signaling pathways, thereby promoting the expression of pro-survival, anti-apoptotic, and inflammatory genes. *APE1*'s multifaceted role may account for *APE1* overexpression being linked to aggressive breast cancer subtypes, especially triple-negative and HER2-enriched tumors [30].

The lack of *APE1* methylation, particularly in the triple-negative and HER2-enriched subtypes, is noteworthy. *APE1* is most likely to support a more aggressive tumor behavior. Figures 3 and 4 provide compelling evidence of the inverse correlation between *APE1* methylation and tumor subtypes' aggressiveness [31].

Comparisons of the A260/A280 spectrophotometric ratios yielded no notable differences among the groups ($P = 0.317$). Therefore, the DNA quality was consistent, and sample integrity may not explain the observed methylation changes.

On the other hand, *APE1* is a key protein in the BER pathway, and its overexpression may alter the response of breast cancer to olaparib and other targeted therapies. *APE1* acts with PARP1 in the repair of DNA single-strand breaks. Therefore, in tumors with overexpression of *APE1*, PARP1 inhibition alone is likely insufficient due to *APE1*'s compensatory role. This underlines the importance of dual targeting of *APE1* and PARP1, particularly in triple-negative breast cancer, where other treatment options are scarce [32].

Also, the greater transcription factor binding apparent in the ChIP assay may suggest that the *APE1* promoter region has an open chromatin structure, which may be associated with the active histone markers H3K9ac and H3K4me3. Future research implementing chromatin accessibility profiling, such as ATAC-seq, and histone modification profiling could shed further light on the epigenetic regulation of *APE1* in the context of breast cancer [33, 34].

Downregulation of *APE1* is associated with worse clinical outcomes. *APE1* expression levels can indicate the presence of breast cancer [18]. Moreover, other studies show that the *APE1* gene, aberrantly expressed in multiple cancers, is a significant locus for atheromatosis and is highly expressed in tumors, and that *APE1* function modifies the effectiveness of PARP1 inhibitors, particularly Olaparib, in breast cancer therapy. The therapeutic population for PARP1 inhibitors is poorly characterized, and more work is needed to understand the role of *APE1* in breast cancer treatment [22].

5. Conclusion

Case analysis also shows that expression levels of *APE1* may be associated with a higher risk of developing breast cancer. Those at risk with unipolar *APE1* gene methylation are less likely to develop breast cancer. A breast cancer patient with a low proliferation score might benefit from *APE1* methylation as a prognostic marker. Low proliferation scores can also reveal the heightened methylation of *APE1*, which semi-positively correlates to risk detection and planning.

Conflicts of interest

There are no conflicts of interest.

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