Apolipoprotein A-1 downregulation promotes basal-like breast cancer cell proliferation and migration associated with DNA methylation

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Abstract. Apolipoprotein A-I (APOA1) performs different roles in different subtypes of breast cancer. It is hypothesized to function as a tumor suppressor in basal-like breast cancer (BLBC). However, the specific role of APOA1 in BLBC and its underlying mechanisms remain unknown. The findings of the present study demonstrated a positive correlation between the expression level of APOA1 and the overall survival of patients with BLBC. Ectopic expression of APOA1 effectively inhibits the proliferation and metastasis of BLBC cells *in vitro*, and these effects are closely related to DNA methylation. To the best of our knowledge, the present study is the first to report increased methylation of the promoter region and decreased methylation of the structural genes of APOA1 in BLBC cells. These alterations resulted in the downregulation of APOA1 expression and suppression of BLBC tumor growth. Collectively, the results of the present study suggested that APOA1 mRNA expression is negatively regulated by DNA methylation in BLBC. Therefore, low expression of APOA1 may be a potential risk biomarker to predict survival in patients with BLBC.

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Introduction

Breast cancer is the prevailing and most lethal form of malignancy afflicting women globally, and is the most common type of cancer, with an estimated 2.3 million new cases in 2020, as well as the leading cause of cancer death among women worlwide, with ~685,000 deaths in 2020, posing a significant threat to public health and wellbeing (1). Based on the expression patterns of 50 specific genes strongly associated with breast cancer (PAM50), it is divided into five intrinsic subtypes including luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) enrichment, basal sample and normal sample (2). Basal-like breast cancer (BLBC) refers to a subtype of triple-negative breast cancer (TNBC), which contributes to 10-20% of breast cancer and is characterized by tumor cells without estrogen receptors (ER), progesterone receptors (PR) or HER2 (3,4). In comparison to ER+/PR+ or HER2+ subtypes, patients with BLBC have the most unfavorable prognosis and highest recurrence rates (5,6). However, due to the absence of hormonal receptors and HER2, targeted therapy options for BLBC are limiting and specific standard treatment regimens are currently unavailable. Hence, identification and understanding the factors and genes implicated in the onset and progression of BLBC is imperative for the management and prognosis of affected individuals.

Dysregulated lipid metabolism is a critical factor in tumor progression (7,8). A previous study revealed the significant involvement of apolipoprotein A-I (APOA1) in various physiological and pathological processes, including but not limited to anti-inflammatory, antioxidant and anti-apoptotic processes (9). In the context of tumor initiation and development, APOA1 has been implicated in the progression of a diverse range of cancers, including breast cancer (10), ovarian cancer (11), colorectal carcinoma (12,13) and liver cancer (14). Despite a wealth of research highlighting APOA1 as a potential biomarker with anti-tumor effects (15,16), the specific biological role and underlying mechanisms of APOA1 in BLBC remain largely unclear.

In the present study, the expression of APOA1 in breast cancer was investigated to analyze its association with BLBC. Subsequently, *in vitro* functional experiments were performed to investigate the biological role of APOA1 in BLBC. To the best of our knowledge, the present study was the first to elucidate the link between the methylation status of APOA1 and gene expression in BLBC cells, providing experimental and theoretical support for using APOA1 as a therapeutic target for BLBC.

Materials and methods

Data acquisition. From the TCGA database (https://portal.gdc.cancer.gov), the TCGA-BRCA project RNAseq data was downloaded, followed by the extraction of the TPM format and clinical data. Additionally, methylation chip data (Illumina450k) for the TCGA-BRCA project was obtained from the UCSC Xena website (https://xenabrowser.net).

Analysis of APOA1 expression level and clinical characteristics. Normal and tumor samples without any clinical information in the database were removed, and the respective expression levels of APOA1 in the transcriptome data were converted numerically according to the log2 (TPM+1) format. Samples were divided into high-APOA1 and low-APOA1 groups based on the median APOA1 expression levels. The differences in clinical characteristics among the two groups were analyzed using R software (4.2.1; https://cran.r-project.org/src/base/R-4/) and the box plot of APOA1 expression between different groups was visualized using the ggplot2 package. $P<0.05$ was considered to indicate a statistically significant difference.

Survival analysis of APOA1 expression. APOA1 expression level, and overall survival (OS) and disease-free survival (DFS) analysis of all breast cancer subtypes and basal subtypes were obtained from the GEPIA website (http://gepia.cancer-pku.cn/) and UCSC Xena (https://xenabrowser.net), respectively. The median of APOA1 expression was used as the cut-off value to distinguish the high and low expression groups of APOA1.

Correlation analysis between APOA1 expression level and methylation value. Samples with transcriptome data and methylation chip detection results were selected from the TCGA-breast cancer project. The data obtained from the UCSC Xena website was utilized to generate a heat map of the methylation of CpG sites and expression levels of the APOA1 gene. Additionally, the scatter plot of these parameters was plotted by Graphpad 8 (https://www.graphpad.com) and analyzed for correlation using Pearson's correlation coefficient). UCSC Xena (https://xenabrowser.net) generated the methylation box diagram of the CpG locus in different breast cancer subtypes.

Plasmids. The pCMV-3Flag plasmid [Elk (Wuhan) Biotechnology, Co., Ltd.] and the following primers were used to create the pCMV-3Flag-APOA1 expression plasmid: Forward, 5'-GATAAAGCCCGGGCGGGATCCATGAAAGCTGCGGTGCTGAC-3'; reverse, 5'-CGACGGTATCGATAAGCTTTCACTGGGTGTTGAGCTTCTTA-3'.

Cell culture and transfection. The BLBC cell lines MDA-MB-468 and MDA-MB-231 (The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences) were cultured in Leibovitz's L15 medium (cat. no. PM151010; Procell Life Science & Technology Co., Ltd.) supplemented with 10% fetal bovine serum (cat. no. 10270-106, Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (cat. no. GNM15140; genom Bio). The MDA-MB-468 and MDA-MB-231 cells were incubated at 37˚C in a sterile incubator without CO_2. Thereafter, cells were transfected with the pCMV-3Flag-APOA1 expression plasmid using Lipofectamine® 2000 *In Vitro* Transfection Reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Specifically, the cells were seeded in a 96-well plate at a density of $1x10^5$ cells/ml and incubated at 37˚C for 24 h. Subsequently, a mixture of pCMV-3Flag or pCMV-3Flag-APOA1 plasmids at a concentration of 2.5 nM and Lipofectamine® 2000 was prepared (total volume, 50 μl) for cell transfection. The cells were cultivated in a 37˚C incubator for 6 h. After which, cells were transferred to complete culture medium, and collected for subsequent detection 24-48 h later.

EdU assay. An EdU Assay Kit (cat. no. C10310-1; Guangzhou Ribobio Co., Ltd.) was used to analyze the cells in the logarithmic growth phase. Following transfection for 24 h, $1x10^5$ cells were seeded in a 6-well plate and incubated overnight. Subsequently, cells were incubated at 37˚C with 100 μl of 50 μM EdU solution for 2 h. The cells were then fixed with 50 μl 4% paraformaldehyde at room temperature for 30 min, followed by neutralization with 50 μl of 2 mg/ml glycine. After permeabilization with 0.5% Triton X-100 for 20 min, cells were stained with 100 μl of 1X Apollo solution for DAPI and 100 μl of 1X Hoechst33342 solution for EdU, both incubated at room temperature for 30 min. Cells were visualized using a fluorescence microscope (Nikon Corporation). Image J software (version 1.50; National Institutes of Health) was used to calculate the proliferative rate in each group.

Cell migration. Transwell chambers without diluted Matrigel were utilized to measure cell migration (cat. no. 3422; Corning, Inc.). A total of 200 μl of serum-free medium was added to the upper chamber and 500 μl of complete culture medium was added to the lower chamber. After transfection for 24 h, cells were prepared in a suspension of $1x10^5$ cells/ml, and 200 μl of cell suspension was added to the upper chamber, followed by an incubation at 37˚C for 24 h. After incubation, the cells were fixed using 4% paraformaldehyde at room temperature for 25 min, followed by crystal violet staining at room temperature for 20 min. Subsequently, the cells in the upper chamber were carefully removed using a cotton swab, while the non-cell seeded side was captured using an inverted microscope.

RT-qPCR. TRIpure Total RNA Extraction Reagent [cat. no. EP013; Elk (Wuhan) Biotechnology Co., Ltd.] was employed to extract RNA from tissues or cells. EntiLink™ 1st Strand cDNA Synthesis Super Mix kit [cat. no. EQ031; Elk (Wuhan) Biotechnology Co., Ltd.] was utilized to reverse transcribe RNA into cDNA, with a reaction temperature of 42˚C for 30 min followed by a 5-min incubation at 85˚C. EnTurbo™ SYBR Green PCR SuperMix kit [cat. no. EQ001; Elk (Wuhan)

Biotechnology Co., Ltd.] was employed in conjunction with the QuantStudio 6 Flex System PCR system (Thermo Fisher Scientific, Inc.) for RT-qPCR. The reaction conditions were as follows: Initial denaturation at 95˚C for 30 sec; denaturation at 95˚C for 10 sec, annealing at 58˚C for 30 sec, extension at 72˚C for 30 sec, repeated for 40 cycles. Gene expression differences were calculated utilizing the relative standard curve and comparative threshold cycle method ($2^{-\Delta\Delta Cq}$) (17), with ACTB and GAPDH as a reference gene. The following primer sequences were used to validate the overexpression efficiency of APOA1: Human APOA1, forward, 5'-CCAGGAGTTCTGGGATAACCT-3', reverse, 5'-GCCACTTCTTCTGGAAGTCGT-3', and human ACTIN, forward, 5'-GTCCACCGCAAATGCTTCTA-3', reverse, 5'-TGCTGTCACCTTCACCGTTC-3'. The following sequences were used to validate the mRNA expression of APOA1 after the addition of 5-AZA: Human APOA1, forward, 5'-CCCTGGGATCGAGTGAAGGA-3', reverse, 5'-CTGGGACACATAGTCTCTGCC-3', and GAPDH, forward, 5'-AGCCACATCGCTCAGACAC-3', reverse, 5'-GCCCAATACGACCAAATCC-3'.

Western blotting. Total protein was extracted using RIPA Lysis Buffer (cat. no. AS1004; Aspen Biological) and Protease Inhibitor Cocktail (cat. no. 04693159001; Roche Diagnostics). The protein concentration was determined using the BCA Protein Quantification Kit (cat. no. AS1086; Aspen Biological). Subsequently, proteins (40 μg/lane) were separated using 10% SDS-PAGE and transferred onto PVDF membranes. The membranes were then blocked with 5% skimmed milk and 0.1% Tween-20 in Tris-buffered saline at room temperature for 1 h. After which, the membranes were incubated overnight at 4˚C with primary antibodies against APOA1 (1:1,000; cat. no. 14427-1-AP; Proteintech Group, Inc.) and β-actin (1:10,000; cat. no. TDY051; Beijing TDY Biotech Co., Ltd.). After washing three times with PBS containing 0.5% Tween-20, the membranes were incubated with HRP-conjugated anti-rabbit secondary antibodies (1:10,000; cat. no. AS1107; Aspen Biological) at room temperature for 1 h. Signal visualization was performed using a Lide110 scanner (Canon, Inc.).

Methylation-specific PCR (MSP). Genomic DNA was extracted from the cultured cells using a gDNA isolation kit [cat. no. EP007; Elk (Wuhan) Biotechnology Co., Ltd.]. Methylation-Gold Kit (cat. no. D5005S; Zymo Research Corp.) was used to convert the genomic DNA into bisulfite-modified DNA. Methylation-specific PCR (MSP) experiments were conducted employing the HieffTM PCR Master Mix (cat. no. 10102ES08; Shanghai Yeasen Biotechnology Co., Ltd.). The unmethylated fragments were amplified using the following APOA1 primers: Forward, 5'-TGGAGTGGGGTGGTTTTAGGGAGT-3', and reverse, 5'-AACCACAACTATTTCTAAACAAAAT-3'. Moreover, the methylated fragments were amplified using the following primers: Forward methylation, 5'-CGGAGCGGGGCGGTTTTAGGGAGT-3', and reverse methylation, 5'-AAACCGCGACTATTTCTAAACGA-3'. The PCR reaction volume was set at 20 μl, and the thermocycling conditions were as follows: Initial denaturation at 98˚C for 4 min, followed by denaturation at 98˚C for 30 sec, annealing at 56˚C for 30 sec, extension at 72˚C for 30 sec and final extension at 72˚C for 10 min. The PCR products were separated by 2% agarose gel electrophoresis, and bands were visualized by staining with GoldView I nucleic acid dye. The concentration of 5-azacytidine (5-AZA; cat. no. HY-A0004; MedChemExpress) in the administration experiment was 10 μM and the experiment was performed three times. Finally, the methylation status of the APOA1 promoter was assessed by analyzing different grayscale values using ImageJ software (version 1.50; National Institutes of Health).

Statistical analysis. R software (4.2.1) (https://cran.r-project.org/src/base/R-4/), GraphPad Prism8 (Dotmatics) and SPSS (version 22; IBM Corp.) were used for statistical analysis and creating figures. A frequency distribution histogram tested the data distribution. In addition, group t-test and one way ANOVA were employed for the statistical analysis between the two groups and multiple groups, respectively. After ANOVA analysis, Bonferroni was used for pairwise comparison. Data without a normal distribution utilized non-parametric tests including Mann-Whitney and Kruskal-Wallis tests were employed for the statistical analysis between two groups and multiple groups, respectively. For *in vitro* experiments, three replicates were set for each group, and the distribution of data in each group was expressed as mean and standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Correlation analysis between APOA1 expression and clinical features. To explore the potential contribution of APOA1 in the progression of breast cancer, the relationship between clinical features and APOA1 mRNA levels in clinical data was analyzed (Table I). A significant correlation between APOA1 expression and ER, PR, HER2, PAM50 and OS was revealed. However, the differences were not statistically significant for all other clinical characteristics.

Expression of APOA1 varies among different breast cancer subtypes. The breast cancer data were further analyzed with different phenotypes in TCGA database and found that ER and PR were positively correlated with APOA1 expression (Fig. 1A and B). However, APOA1 expression was lower in the HER2+ group relative to the HER2-group (Fig. 1C). Simultaneously, the APOA1 expression in the lumina subtype was markedly elevated compared with that in the basal and HER2 subtypes (Fig. 1D), indicating its diverse biological roles in different phenotypes of breast cancer.

APOA1 is an effective prognostic factor for breast cancer and patients with BLBC. To further investigate the involvement of APOA1 in BLBC, the correlation between APOA1 expression and OS and DFS using TCGA database was examined. The Kaplan Meier survival analysis showed that the APOA1 down-regulation was associated with poor prognosis in patients with breast cancer and BLBC (Fig. 2A and B). Meanwhile, the association between ApoAI expression and DFS in breast cancer patients was explored. The analysis revealed a trend consistent with the OS findings, suggesting that higher levels of ApoAI expression may correlate with improved DFS outcomes (Fig. 2C and D). However, it is important to note that

Table I. Clinical analysis results of high- and low-APOA1 groups.

Characteristic	Low expression of APOA1	High expression of APOA1	P-value	Statistical test
n	541	542		
T stage, n (%)			0.166	Chi square
T1	132 (12.2)	145 (13.4)		
T2	328 (30.4)	301 (27.9)		
T3	60 (5.6)	79 (7.3)		
T4	20 (1.9)	15 (1.4)		
N stage, n (%)			0.459	Chi square
N0	250 (23.5)	264 (24.8)		
N1	173 (16.3)	185 (17.4)		
N2	62 (5.8)	54 (5.1)		
N3	43 (4)	33 (3.1)		
M stage, n (%)			0.350	Chi square
M0	468 (50.8)	434 (47.1)		
M1	13 (1.4)	7 (0.8)		
Pathologic stage, n (%)			0.448	Chi square
Stage I	85 (8)	96 (9.1)		
Stage II	310 (29.2)	309 (29.2)		
Stage III	121 (11.4)	121 (11.4)		
Stage IV	12 (1.1)	6 (0.6)		
Ethnicity, n (%)			0.246	Chi square
Asian	35 (3.5)	25 (2.5)		
Black or African American	83 (8.4)	98 (9.9)		
White	369 (37.1)	384 (38.6)		
Age, n (%)			0.152	Chi square
≤60	288 (26.6)	313 (28.9)		
>60	253 (23.4)	229 (21.1)		
Histological type, n (%)			<0.001	Chi square
Infiltrating ductal carcinoma	421 (43.1)	351 (35.9)		
Infiltrating lobular carcinoma	72 (7.4)	133 (13.6)		
PR status, n (%)			<0.001	Fisher's
Negative	208 (20.1)	134 (13)		
Indeterminate	3 (0.3)	1 (0.1)		
Positive	303 (29.3)	385 (37.2)		
ER status, n (%)			<0.001	Fisher's
Negative	158 (15.3)	82 (7.9)		
Indeterminate	2 (0.2)	0 (0)		
Positive	354 (34.2)	439 (42.4)		
HER2 status, n (%)			0.025	Chi square
Negative	265 (36.5)	293 (40.3)		
Indeterminate	8 (1.1)	4 (0.6)		
Positive	92 (12.7)	65 (8.9)		
PAM50, n (%)			<0.001	Chi square
Normal	20 (1.8)	20 (1.8)		
LumA	230 (21.2)	332 (30.7)		
LumB	102 (9.4)	102 (9.4)		
Her2	68 (6.3)	14 (1.3)		
Basal	121 (11.2)	74 (6.8)		

Table I. Continued.

Characteristic	Low expression of APOA1	High expression of APOA1	P-value	Statistical test
Menopause status, n (%)			0.760	Chi square
Pre-	114 (11.7)	115 (11.8)		
Peri-	19 (2)	21 (2.2)		
Post-	365 (37.6)	338 (34.8)		
PFI event, n (%)			0.470	Chi square
Alive	463 (42.8)	473 (43.7)		
Deceased	78 (7.2)	69 (6.4)		
DSS event, n (%)			0.100	Chi square
Alive	478 (45)	500 (47)		
Deceased	50 (4.7)	35 (3.3)		
OS event, n (%)			0.011	Chi square
Alive	450 (41.6)	481 (44.4)		
Deceased	91 (8.4)	61 (5.6)		
Radiation therapy, n (%)			0.579	Chi square
No	208 (21.1)	226 (22.9)		
Yes	276 (28)	277 (28.1)		
Anatomic neoplasm subdivisions, n (%)			0.928	Chi square
Left	280 (25.9)	283 (26.1)		
Right	261 (24.1)	259 (23.9)		
Age, median (IQR)	59 (49, 68)	58 (48, 67)	0.277	Wilcoxon

APOA1, apolipoprotein A-I; ER, estrogen receptor; PR, progesterone receptor; Her2, human epidermal growth factor receptor 2; LumA, luminal A; LumB, luminal B; PFI, Progression-Free Interval; DSS, disease-specific survival; OS, overall survival; IQR, interquartile range.

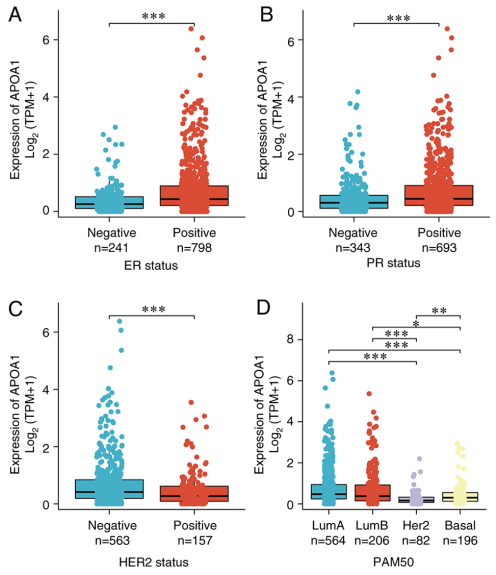

Figure 1. Expression of APOA1 varies among different breast cancer subtypes. (A) APOA1 expression levels were markedly elevated in the ER+ subtype. (B) APOA1 expression levels were significantly increased in the PR+ subtype. (C) APOA1 expression levels showed a notable elevation in the HER2- subtype. (D) APOA1 expression levels were markedly increased in the luminal subtype compared with basal and HER2 subtypes. The data are presented as the median (upper quartile-lower quartile). ***P<0.001, **P<0.01, *P<0.05. APOA1, apolipoprotein A-I; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; LumA, luminal A; LumB, luminal B.

this trend did not reach statistical significance, likely due to the limitations posed by sample size and potential confounding variables.

APOA1 inhibits the proliferation and migration of BLBC cells in vitro. To evaluate whether APOA1 participates in the development of BLBC, four transfectants, MDA-MB-468 (APOA1), MDA-MB-468 (Vector), MDA-MB-231 (APOA1), and MDA-MB-231 (Vector), were constructed and examined by RT-qPCR (Fig. S1). The effect of APOA1 on the proliferation of BLBC cells was studied *in vitro*. Results of the EdU assay showed that APOA1 significantly inhibited DNA replication within the BLBC cells (Fig. 3A-D). Furthermore, transwell assay confirmed that APOA1 inhibited the migration of BLBC cells (Fig. 3E-H). These results suggested that APOA1, as a BLBC suppressor gene, may inhibit the proliferation and migration of BLBC cells *in vitro*.

APOA1 mRNA expression level is negatively correlated with APOA1 methylation level in breast cancer cells. The UCSC Xena website was used to generate a comparative heatmap (Fig. 4A). As depicted in Fig. 4B, the expression of APOA1 mRNA was significantly down-regulated due to the methylation of its promoter. Further analysis focused on the correlation between the methylation of individual CpG sites and APOA1 expression levels. Specifically, a significant negative correlation was observed at four out of six methylation

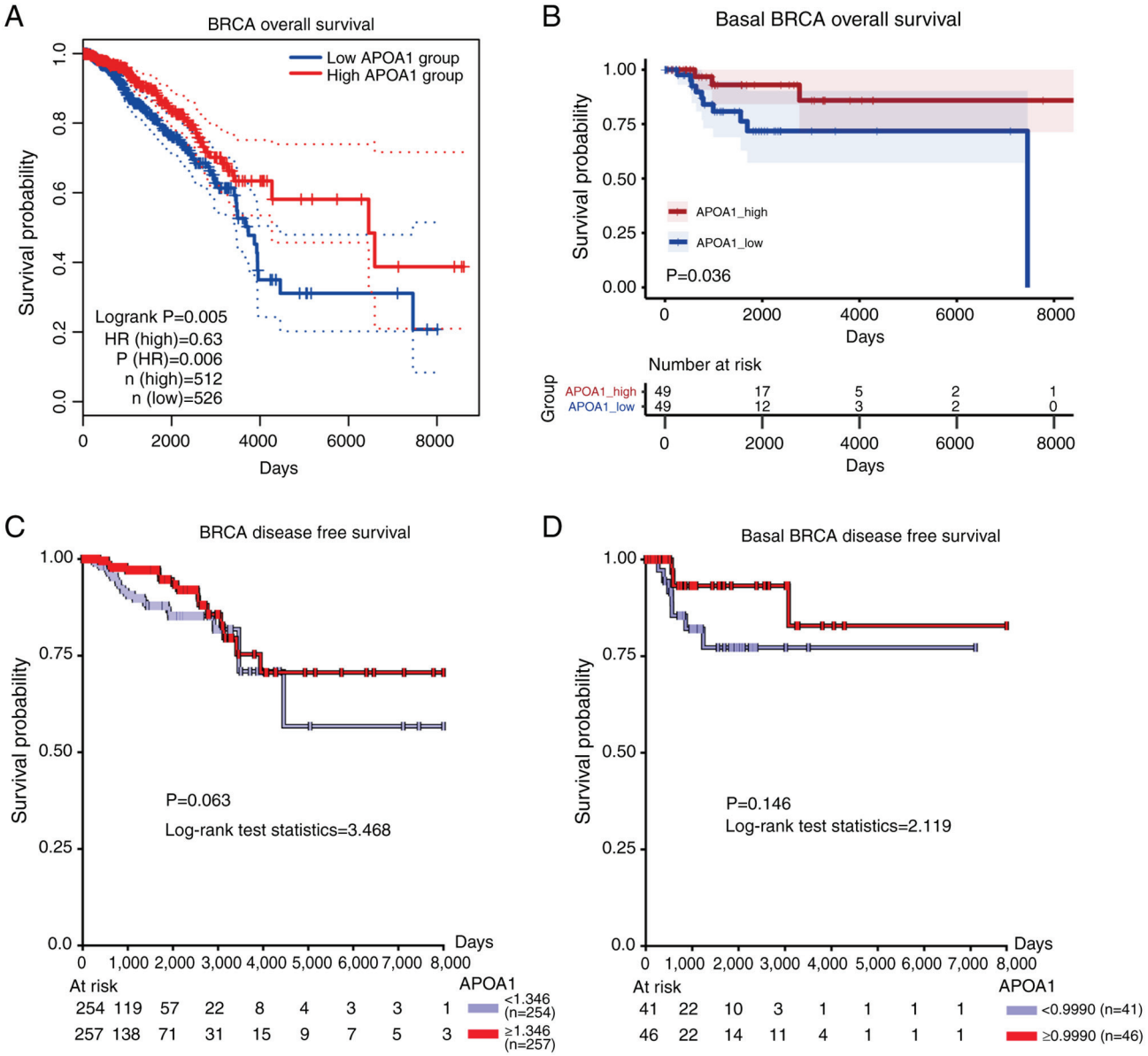

Figure 2. APOA1 is an effective prognostic factor for BRCA and patients with basal-like breast cancer. (A) Association between APOA1 expression levels and the overall survival of patients with BRCA. (B) Association between APOA1 expression levels and the overall survival of patients with BRCA and basal-like breast cancer. (C) Association between APOA1 expression levels and the disease-free survival of patients with BRCA. (D) Association between APOA1 expression levels and the disease-free survival of patients with BRCA and basal-like breast cancer. APOA1, apolipoprotein A-I; BRCA, breast cancer; HR, hazard ratio.

levels: cg26734040, cg19324627, cg03010018 and cg24984312 (Fig. 4C-H showing six CpG sites near the APOA1 promoter). In contrast, no statistical differences were observed in the APOA1 expression at the cg03856801 sites. Although, APOA1 expression at cg25987102 was statistically significant, the correlation coefficient r was low (r=-0.1280), and considering that this site was far from the APOA1 gene promoter region, it was not considered in subsequent analysis.

APOA1 methylation level is different in different subtypes of breast cancer. The distribution map of the APOA1 CpG site also indicated significant differences in APOA1 methylation levels between different subtypes of breast cancer, except for the cg25987102 site. Furthermore, the basal subtype exhibited significantly higher methylation levels at four CpG sites, including cg26734040, cg19324627, cg0301018, and cg24984312. Notably, significantly reduced methylation levels were observed for cg19360562, cg00142925 and cg10753889 (Fig. 5). These results implied that DNA methylation may be one of the molecular mechanisms regulating APOA1 expression in BLBC.

5-AZA reverses APOA1 expression in BLBC cells. To verify the hypothesis, the effect of 5-AZA on APOA1 gene expression in the BLBC cell lines MDA-MB-468 and MDA-MB-231 was investigated. The results demonstrated that the levels of APOA1 mRNA and protein expressions were elevated in the presence of 5-AZA (Fig. 6A-C). In addition, APOA1 gene methylation and subsequent expression in MDA-MB-468 and MDA-MB-231 cells treated with 5-AZA was examined. The

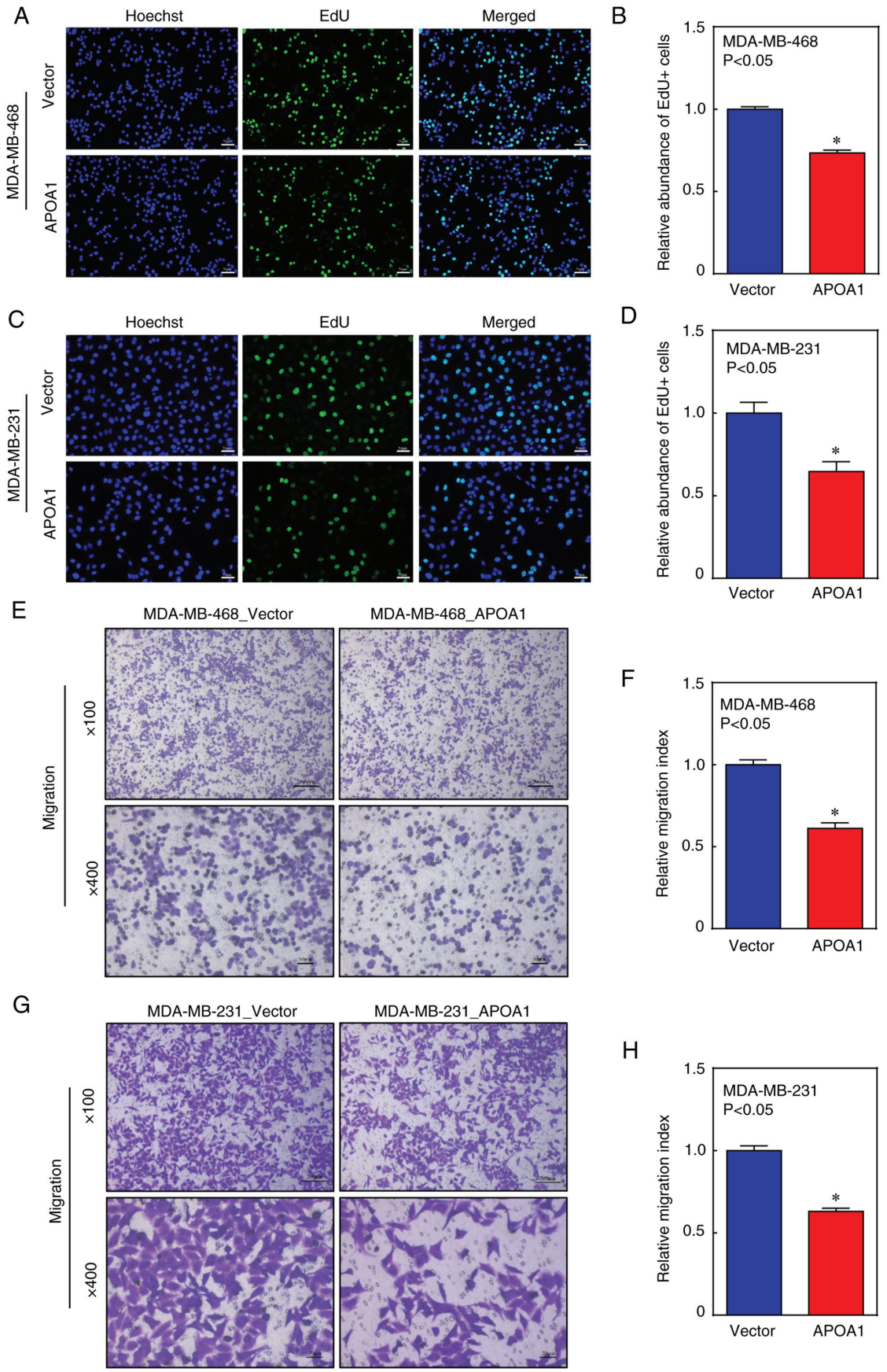

Figure 3. APOA1 inhibits the proliferation and migration of basal-like breast cancer cells *in vitro*. (A) EdU assay to examine the impact of APOA1 on the DNA replication activity of MDA-MB-468 cell and (B) the results quantified. (C) EdU assay to examine the impact of APOA1 on the DNA replication activity of MDA-MB-231 cells and (D) the results quantified. (E) Transwell assay to determine the effect of APOA1 overexpression on MDA-MB-468 cells and (F) analysis of relative migration index. (G) Transwell assay to determine the effect of APOA1 overexpression on MDA-MB-231 cells and (H) analysis of relative migration index. Scale bar, migration x100: 200 μm, all others 50 μm. Student's t-test was used to analyze the data and presented as the mean + SD (Unpaired, *P<0.05). APOA1, apolipoprotein A-I; LumA, luminal A; LumB, luminal B.

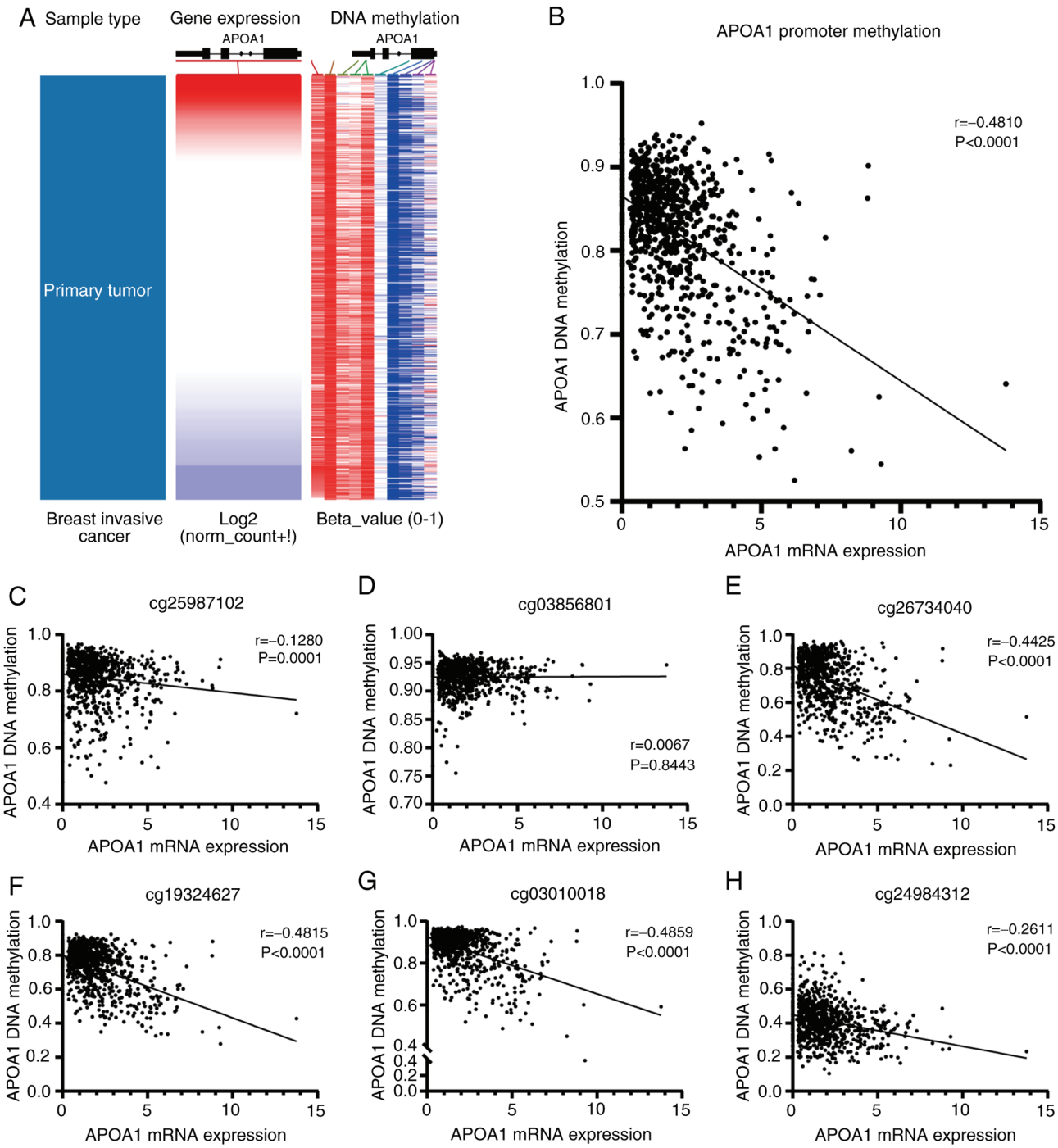

Figure 4. APOA1 mRNA expression level was negatively correlated with APOA1 methylation in breast cancer cells. (A) Heatmap of APOA1 expression and methylation levels in breast cancer tumor tissue from the UCSC Xena database. (B) Correlation between APOA1 expression and methylation in breast cancer. (C) Correlation analysis between the expression level of APOA1 and the methylation of CpGsite cg25987102, (D) cg03856801, (E) cg26734040, (F) cg19324627, (G) cg03010018 and (H) cg24984312 near the APOA1 promoter region. APOA1, apolipoprotein A-I.

results showed that the methylation level of APOA1 decreased significantly after the addition of 5-AZA (Fig. 6D-G). These results indicated that 5-AZA could reduce APOA1 methylation level, thereby promoting APOA1 expression.

5-AZA inhibits cell proliferation and migration of BLBC cells. As shown in Fig. 7A-D, EdU+ cells were significantly decreased in 5-AZA-treated cells. In addition, the transwell assay showed that the migration ability of these cells was significantly reduced after 5-AZA treatment (Fig. 7E-H). These findings suggested that 5-AZA treatment modulated the proliferation and invasion ability of BLBC cells, which was related to the induction of APOA1.

Discussion

The global incidence of breast cancer has been consistently increasing, rendering it a predominant malignancy among women (18). With a high early recurrence rate and frequent distant metastasis, BLBC often manifests as a high-grade

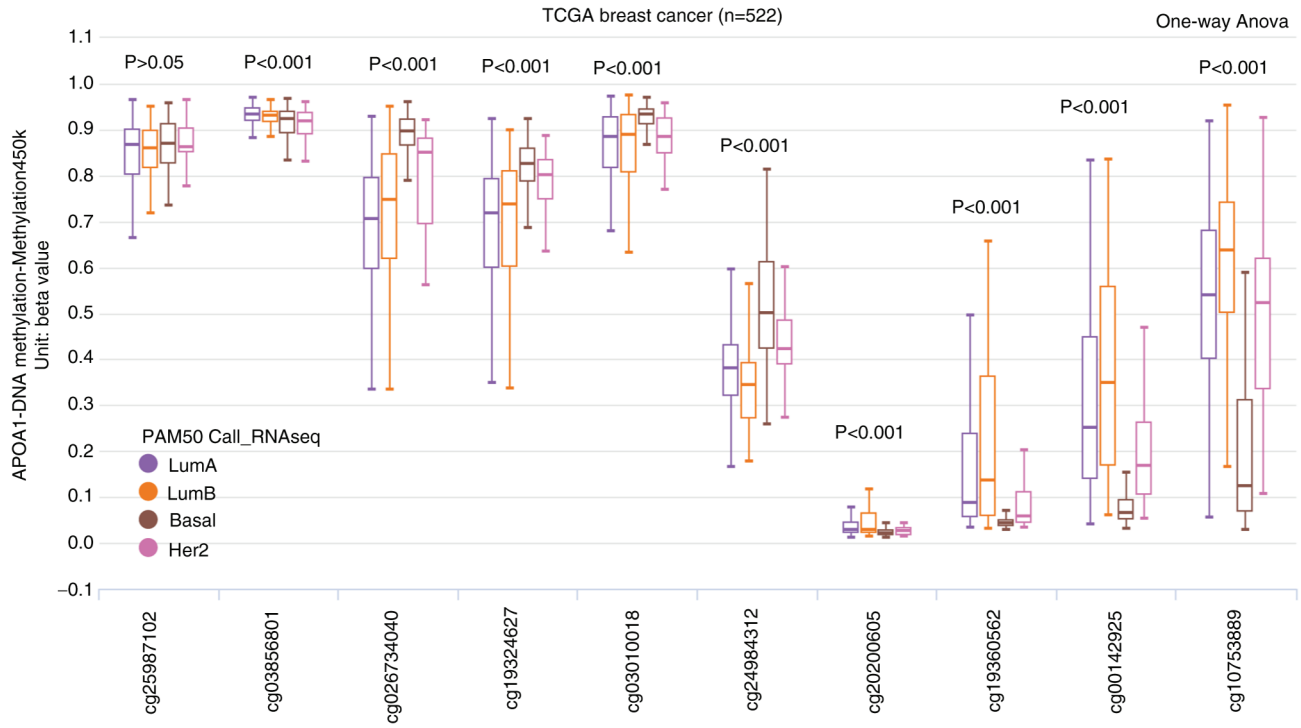

Figure 5. APOA1 methylation level varies in different subtypes of breast cancer. APOA1, apolipoprotein A-I; Her2, human epidermal growth factor receptor 2.

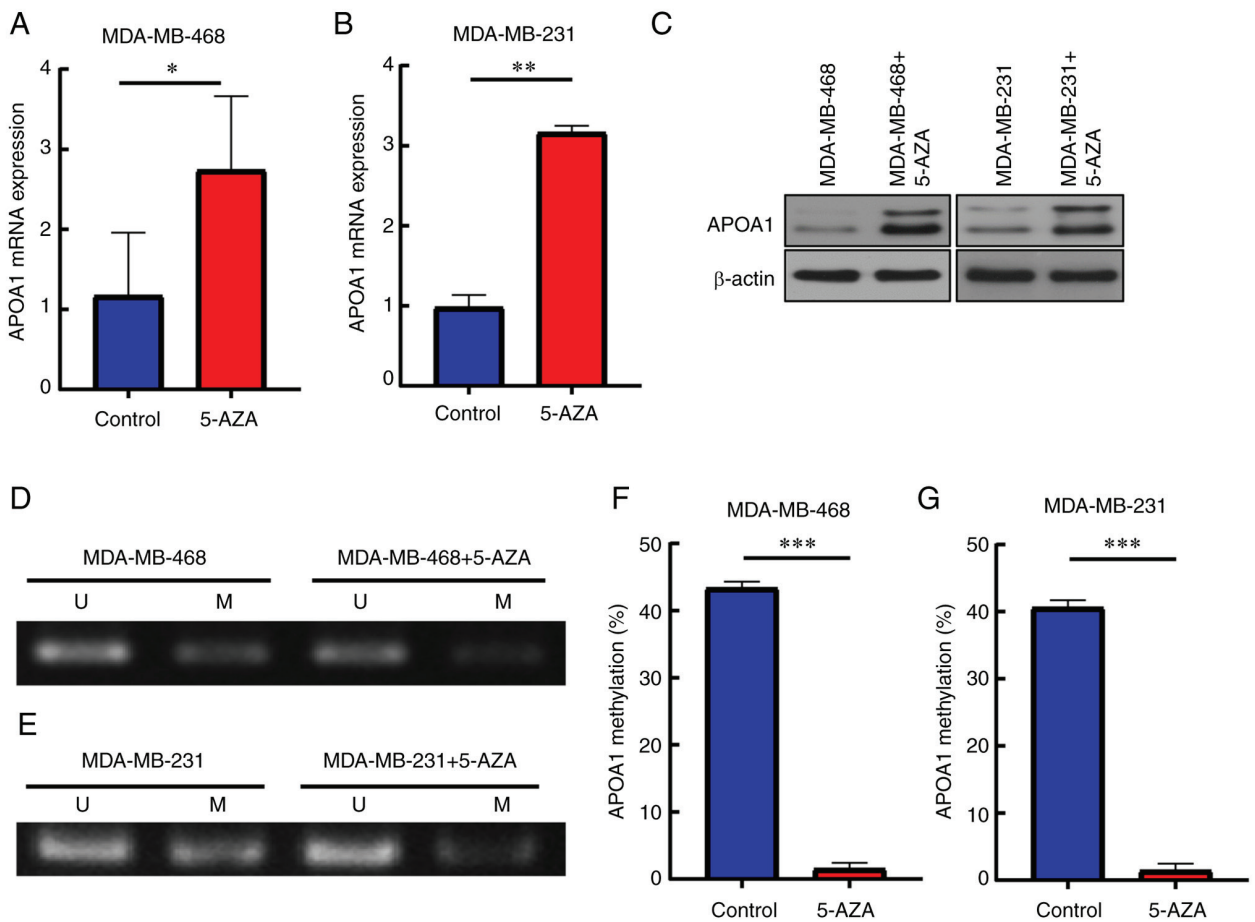

Figure 6. 5-AZA reverses APOA1 expression in BLBC cells. (A) RT-qPCR analysis of APOA1 mRNA in MDA-MB-468 and (B) MDA-MB-231 cells following 5-AZA application. (C) Western blotting to detect the APOA1 protein level in BLBC cells after 5-AZA treatment. (D) Demethylation of APOA1 in MDA-MB-468 cells by 5-AZA and (E) the results quantified. (F) Demethylation of APOA1 in MDA-MB-231 cells and (G) the results quantified. by 5-AZA. Data were presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01, ***P<0.001. M, methylated product; U, unmethylated product; APOA1, apolipoprotein A-I; BLBC, basal-like breast cancer; 5-AZA, 5'-AZAcytidine.

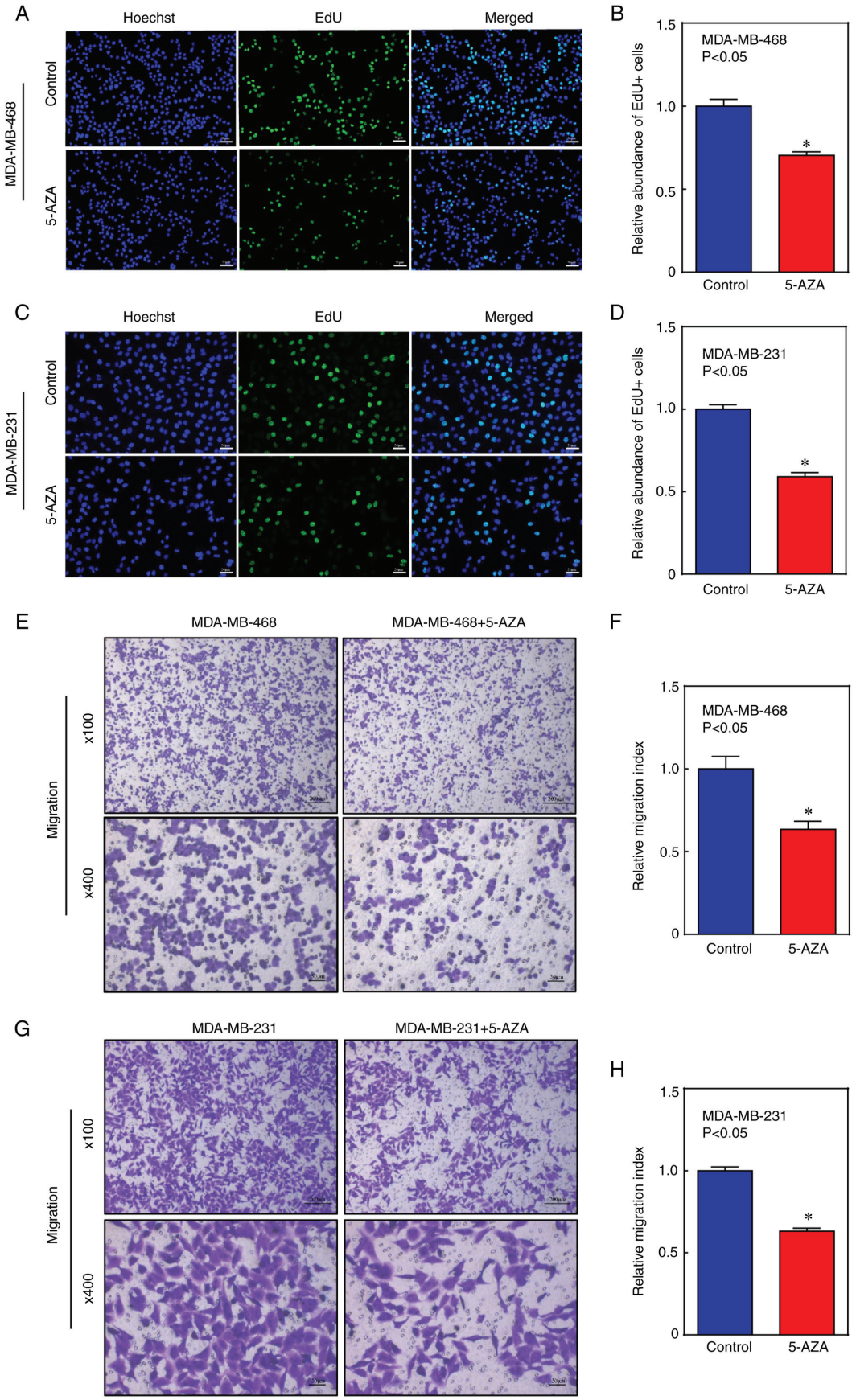

Figure 7. 5-AZA inhibits cell proliferation and migration of BLBC cells. (A) EdU assay to examine the impact of 5-AZA on the DNA replication activity of MDA-MB-468 cells and (B) the results quantified. (C) EdU assay to examine the impact of 5-AZA on the DNA replication activity of MDA-MB-231 cells and (D) the results quantified. (E) Transwell assays to determine the effect of 5-AZA on MDA-MB-468 cells and (F) analysis of relative migration index. (G) Transwell assays to determine the effect of 5-AZA on MDA-MB-231 cells and (H) analysis of the relative migration index. Scale bar, migration x100: 200 μm, all others 50 μm. Unpaired Student's t-test was used to analyze the data and the values were presented as the mean + SD (*P<0.05). 5-AZA, 5'-AZAcytidine; BLBC, basal-like breast cancer; APOA1, apolipoprotein A-I.

invasive ductal carcinoma, compared with the other subtypes of breast cancer (including luminal A, luminal B, HER2-enriched), and has a poor prognosis (6). Despite advancements in tumor biology, the clinical results for patients with BLBC remain unsatisfactory; therefore, there is an urgent need to develop more effective therapeutics for its treatment (19). A substantial amount of evidence has proven that biomarkers and detectable markers are effective for the prediction and treatment of diseases (20-22). Hence, the present study investigated the clinical feasibility of using APOA1 for the prognosis of survival in patients with BLBC. The present study showed that the expression of APOA1 was not directly related to traditional tumor staging parameters (T, N, M stages and pathological stages) and the association of APOA1 levels with OS suggests that APOA1 may exert its influence on prognosis through broader mechanisms. Similar observations have been reported in other studies (23,24). Such broader mechanisms include affecting the tumor microenvironment, immune response or specific signaling pathways.

APOA1 is abnormally expressed in various malignancies. For instance, in renal clear cell carcinoma, poor OS and DFS are linked to increased APOA1 mRNA levels (25). Reportedly, APOA1 functions as a tumor suppressor, and its reduced methylation can be considered a promising prognostic marker in liver cancer (26). In colorectal cancer, decreased serum levels of APOA1 are linked to higher staging, systemic inflammation and poor patient prognosis (12,13). The present study revealed that HER2-enriched and BLBC subtypes exhibited lower levels of APOA1, suggesting its specific role in the disease progression. In the present study, the expression level of APOA1 aligns with the aggressive characteristics and poor prognosis associated with HER2 and BLBC subtypes. These findings underscore the importance of further research into APOA1 as an independent prognostic biomarker and its function in the subtype-specific pathophysiology of breast cancer. Furthermore, the present research results also indicated that APOA1 inhibits the proliferation and migration of MDA-MB-468 and MDA-MB-231 cells *in vitro*. Furthermore, the present study demonstrated a lack of correlation with tumor stage, suggesting the complexity of the function of APOA1 in BLBC progression, suggesting a multifaceted role beyond conventional tumor staging markers. It was hypothesized that APOA1 may exert its influence on tumor cell migration through intricate intracellular signaling pathways or interactions within the tumor microenvironment. This provides novel insights into the nuanced role of APOA1 in BLBC pathology and emphasizes the importance of further research to elucidate its specific mechanisms of action and clinical implications. However, the relationship between APOA1 expression level and tumor size *in vivo* requires investigation and these topics will be addressed in future research.

DNA methylation is a prevalent epigenetic phenomenon (27,28). In normal conditions, CpG sites can congregate on CpG islands. The CpG islands in the gene promoter region are typically unmethylated. However, the level of methylation may fluctuate as a result of the genetic regulatory processes underlying diverse pathological disorders (29,30). According to previous studies, low expression of APOA1 in renal cell carcinoma may result from increased DNA methylation of the APOA1 gene (25) and Guo *et al* (26) revealed that patients with liver cancer with high levels of APOA1 DNA methylation had significantly longer progression-free survival time than those with low methylation. However, the correlation between DNA methylation and APOA1 expression level in BLBC has not been reported yet. To the best of our knowledge, the present study was the first to report that increased methylation of APOA1 promoter and decreased methylation of its structural genes affect the expression level of APOA1. This is consistent with various studies reporting that DNA methylation near the transcription start site usually inhibits gene expression. However, the methylation in structural genes has a reverse impact (31-35).

Demethylating drugs have demonstrated efficacy in treating hematological malignancies and solid tumors (36,37). According to previous studies, treatment with azacitidine or decitabine increased the sensitivity of patients with ovarian cancer to carboplatin (38-40). Moreover, patients treated with azacitidine showed an improved response to anti-cancer therapy against non-small cell lung cancer (41). In BLBC, combined therapy with 6-mercaptopurine and 5-AZA makes the cells more sensitive to chemotherapeutic drugs; this treatment is suitable before disease recurrence and can potentially inhibit TNBC cells with high drug resistance (42). This indicates that the discovery of new biomarkers for predicting treatment responses, novel methylation inhibitors and/or combined therapeutic strategies to target specific processes underlying tumor development is an important research avenue to achieve the best therapeutic effect in BLBC.

In conclusion, the present study reported that downregulated APOA1 expression was associated with a shortened OS in patients with breast cancer. Furthermore, it was demonstrated that APOA1 could inhibit the proliferation and migration abilities of BLBC cells. Notably, DNA methylation could significantly affect the expression level of APOA1. This evidence supported that APOA1 has a significant role in the proliferation and migration of BLBC cells and that APOA1 and specific inhibition of its methylation could be potential targets for the treatment of BLBC, thus providing a theoretical basis for the targeted therapy of BLBC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

CW, ZZ and SY designed the research study. CW, SC, RZ and MC performed the research. XY, YH, ZS and QM participated in the statistical analysis and had input in the experimental design. CW and SY wrote the main manuscript text. XY, YH, ZS and QM confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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