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ORIGINAL RESEARCH

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Promoter's hypermethylation and down-regulated expression of amphiregulin in breast carcinoma: a potential prognostic marker

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Abstract

To evaluate the role of amphiregulin (AREG), a ligand of epidermal growth factor receptor, in breast invasive carcinoma (BRIC) the present study was initiated. For this purpose, we used freely available GeneCards Suite for AREG enrichment analysis, the online UALCAN web portal for analysis of differential expression and promoter's methylation status of AREG, the online cBioPortal cancer genome atlas to document carcinoma-associated AREG mutations and CCLE (Cancer Cell Line Encyclopedia) and GDSC (Genomics of Drug Sensitivity in Cancer) toolkit to document AREG's sensitivity towards various anti-cancer drugs. We observed lower expression of AREG in the majority of patients, in comparison to normal controls, due to promoter's hypermethylation. While AREG's upregulation was examined in premenopausal condition, stage 1 and a few other patient groups. The reduced expression of AREG in human epidermal growth factor receptor 2 (HER2) positive, triple-negative and patients of many other subtypes highlights the relevance of AREG with the progression of the disease. Moreover, the AREG's expression improved the effectiveness of anti-neoplastic drugs but confers resistance against gamma-secretase inhibitors. Patients exhibiting lower/medium levels of AREG's expression had better survival chances. In the future, an investigation of the factors which modulate methylation patterns of AREG's promoter and an evaluation of the prognostic power of AREG's expression will facilitate the identification of novel therapeutic channels for better prognosis of BRIC and devise a multilayered treatment strategy.

Keywords

Amphiregulin (AREG); Invasive lobular carcinoma (ILC); Triple-negative breast carcinoma (TNBC); Gene expression; Promoter methylation; Survival analysis

1. Introduction

The taxonomic studies have classified breast cancer (BC) on the basis of initial primary site's localization and dispersed distant tissue localization as *in situ* and invasive carcinoma, respectively [1]. Similarly, there are main two histological divisions, *i.e.*, Ductal carcinoma *in situ* (DCIS)/invasive ductal carcinoma (IDC) and Lobular carcinoma *in situ* (LCIS)/invasive lobular carcinoma (ILC). DCIS has five subtypes including papillary, micropapillary, cribriform, solid and comedo [2]. On the basis of expression status of receptors BC is classified into Luminal A (estrogen/progesterone receptor positive with HER2 negative), Luminal B Estrogen receptor (ER) and progesterone receptor (PR) (ER/PR positive with HER2+/−), HER2 positive (strong positive expression of HER2), Basal-like (positive expression of cytokeratins) and triple-negative breast carcinoma (TNBC) (all receptors, *i.e.*, ER, PR and HER2 are negatively correlated here) [3]. TNBC is a worse malignancy which has a collection of somatic mutations that promote invasiveness [4, 5].

Amphiregulin (AREG), a family member of an epidermal growth factor (EGFA) family, is associated with the surface of activated monocytes [6, 7]. AREG is released after the proteolytic cleavage of pro-AREG transmembrane precursor by tumor-necrosis factor-alpha converting enzyme which is a family member of ADAM (A Disintegrin and Metalloprotease) family [8, 9]. AREG-EGFR (Epidermal growth factor receptor) association triggers a group of cellular signaling events regarding cell survival, proliferation and metabolism [10]. Various reports have shown the role of AREG in epithelial carcinomas' resistance to apoptosis [11, 12]. Moreover, AREG has an influential role in respiratory diseases and rheumatoid arthritis like inflammatory complications [13]. The TNF (tumor necrosis factor) receptor family members mediate associations of specific transmembrane receptors for the induction of extrinsic apoptosis signaling events [14]. FasL and FasR are important members of the TNF family that constitute death-inducing complexes through the binding of adaptor proteins

with receptors via death domains and mediate activation of pro-caspase 8 [15, 16]. The active caspase 8 leads to the critical deploy the execution phase of apoptosis by promoting the interaction of FasL with cell membrane growth factors [17–19]. AREG strengthens tumor progression by blocking apoptosis and binding with FasL in several cancers [20].

AREG plays significant role in mammary gland's development, homeostasis, lungs' morphogenesis and proliferation of keratinocytes [21, 22]. AREG's expression has an essential role in maintaining tissue integrity, regulating functioning of CD8+ tumor-infiltrating T cells, and facilitating FoxP3-CD4+ pathway-mediated repair of colon's muscles [23]. To the best of our knowledge we are the first to conduct multi-layered *in-silico* analysis of AREG's expression, mutation, methylation and drug sensitivity data to evaluate the potential role of AREG, if any, in the pathogenesis of multi factorial breast invasive carcinoma (BRIC).

2. Materials and methods

2.1 AREG enrichment exploration

AREG enrichment analysis was done using GeneCards Suite (available at: www.genecards.org) a meta-database that is collection of omics techniques, *i.e.*, genomics, transcriptomics, proteomics, metabolomics and clinical information. The suit encompasses 21,137 genes, 22,237 pseudogenes, 500 hot genes, 14 gene clusters and 13,686 biological diseases. The suit has "search-box" in which we queried the AREG gene and pressed "GO" function that displayed various attributes including gene aliases, exome structure, domains view, protein interaction motifs, molecular interactors, mutation-mediated diseases and drugs.

2.2 Investigation of differential expression of AREG transcripts

AREG gene expression analysis in normal and cancerous breast tissue and its association with several other parameters, *i.e.*, stage, race, age, gender, subclasses, histological types and menopause status was carried out using UALCAN (https://ualcan.path.uab.edu/). The UALCAN [24] uses the cancer genome atlas (TCGA) RNA-seq level 3 data of approximately more than 31 malignancies. The database has some functional options, *i.e.*, first we pressed the "Analysis" option that showed the "search-box" for AREG querying in the Breast Invasive Carcinoma dataset. The database has an "Explore" option that displayed graphic or whisker box plot representation of AREG differential expression results. The database uses quartile ranges from minimum to maximum values that indicate differential expression, *i.e.*, ranging from lower to higher, respectively. Expression of transcripts was measured as the number of transcripts per million (TPM). Detail of the BRIC samples in TCGA datasets used for transcripts expression analysis is mentioned in **Supplementary Table 1**.

2.3 Investigation of differential expression of AREG protein

The online web portal UALCAN (https://ualcan.path.uab.edu/) allows us to investigate AREG differential expression at both transcriptomic and proteomic levels. It further helps us to correlate levels of transcripts and encoded proteins with various aspects of breast cancer including AREG expression in primary tumors and patients of different breast cancer (BRCA) stages, races, age groups, subclasses and histological types. The web portal integrates mass spectrometry-based proteomic data from The Clinical Proteomic Tumor Analysis Consortium (CPTAC) in various cancer types. To have an account of AREG encoded protein's expression we carried out CPTAC analysis. The information of the BRIC samples used for AREG protein expression analysis is mentioned in **Supplementary Table 2**.

2.4 AREG promoter methylation scrutiny

AREG promoter methylation status was determined through the online UALCAN (https://ualcan.path.uab.edu/) database that contains methylome data of 31 cancer types. The database links gene expression with methylation status either hypo or hyper based on beta values, *i.e.*, 0 for fully unmethylated state and 1 for fully methylated state. The database uses cut-off values for hypo-methylation (0.3–0.2) and hyper-methylation (0.7–0.5). The detail of the BRIC samples used for AREG's promoter methylation analysis is given in **Supplementary Table 3**.

2.5 AREG mutatome map mining

AREG mutatome map retrieval was carried out using the cBioPortal cancer genome server (www.cbioportal.org) which is a huge repository of cancer genome data. We selected the "Breast Invasive Carcinoma TCGA, Cell 2015" dataset by querying AREG in the gene "search-box" and finally pressed the "submit" option that displayed a map of mutations from the "mutation" option. The map covers mutation types, positions and frequency in the AREG gene with the cancer dataset. The detail of the BRIC samples used for AREG's mutatome analysis is provided in **Supplementary Table 4**.

2.6 AREG drug sensitivity inquiry

AREG dug effectiveness analysis was done through an online user-friendly CCLE GDSC toolkit (https://public.tableau.com/app/profile/jason.roszik/viz/CCLE_GDSC_correlations/CCLE_GDSC) that is a wide range consortium of gene expression correlations with drug effectiveness, *i.e.*, enhancing or resisting towards drug efficiency. We used AREG as a query gene in the "gene list" option and adjust coefficient concentrations as "EC50/IC50" values. The toolkit uses a coloring pattern to represent enhancing or resisting behaviors of genes, *i.e.*, red (positive with resistance) and green (negative with enhancing the drug effect).

2.7 Kaplan Meier survival curve analysis

We explored the association of patient's survival (*i.e.*, days to death or last follow-up time) during treatment and differential expression states (high, medium and low) of AREG by Kaplan-Meier (KM) plots using the online web portal UALCAN (https://ualcan.path.uab.edu/). The UALCAN integrates patient's survival TCGA data through the systemic pipeline. KM plot curves related duration of patients' survival, AREG expression status, and different variables, *i.e.*, race, gender and cancer subtypes based on log-rank test algorithm.

3. Results

This study involved *in-silico* analysis of data available in the TCGA database submitted by various wet laboratories worldwide. This analysis helped to establish the AREG profile and enhanced understanding regarding the contribution of AREG in the pathogenesis of BRIC.

3.1 AREG enrichment analysis

AREG is also known as Colorectum Cell-Derived Growth Factor and Schwannoma-Cell Derived Growth Factor. AREG is a protein-coding gene located at chromosome 4q13.3 and consists of 9912 base pairs (ranging from 74,495,098–74,455,009 nucleotides) which encode a protein composed of 252 amino acids having a molecular mass of 27,895 Da. The AREG protein is mainly located in the extracellular part and nucleus and is known for its participation in ovarian and colorectal malignancies. AREG is an autocrine growth factor that belongs to the epidermal growth factor family and is involved in the promotion of the cell cycle. It prevents the growth of cancer cell lines and has a significant role in bone tissue, oocyte and breast tissue development. It enhances the activity of the EGFR and ERBB2 (Erb-b2 receptor tyrosine kinase 2) and is activated by SRC (a nonreceptor tyrosine kinase), WT1 (Wilms tumor protein, WT1 Transcription Factor), ADAM17 (A disintegrin and metalloprotease 17) and F2RL1 (F2R like trypsin receptor 1) and inactivated by STAB1 (Stabilin 1). It is also involved in PI3K (phosphoinositide 3-kinase)-Akt (protein kinase B), MAPK (Mitogen-activated protein kinase), Hippo and ErbB signaling pathways (Table 1).

3.2 Differential expression analysis of AREG transcripts

AREG exhibited lower expression in a majority of the breast carcinoma patients as compared to the normal subjects. While male patients were observed to have AREG levels higher than observed in normal individuals. Breast cancer stages indicate the progression of the disease into nearby tissues and distant localizations. AREG expression was highest in stage 1 patients (133.23 TPM) followed by stage 3 (123.263 TPM), stage 2 (88.84 TPM) and stage 4 (80.48 TPM) patients. But AREG expression in patients of different stages varied insignificantly ($p > 0.05$) as compared to normal expression level (164.12 TPM). AREG has diverse expression in various world populations including Caucasian (114.21 TPM), African-American (60.61 TPM) and Asian (71.94 TPM). The male patients had higher AREG expression (242.12 TPM) as compared to normal subjects and female patients (101.69 TPM) that showed the impact of the male endocrine system on AREG expression. Although AREG expression is reduced in most of the patients, the stage of cancer, the patients' race and the patients' gender seemed to have no significant role ($p > 0.05$) in lowering the expression of AREG. It was further noticed that AREG possessed higher expression in patients of 21–40 years (190.09 TPM) as compared to 41–60 years (134.371 TPM), 61–80 years (66.44 TPM) and 81–100 years (80.48 TPM). The patients of age 21–40 years and 81–100 years exhibited significant variations ($p < 0.05$) in normal controls. While variations among all patient groups were significant ($p < 0.05$) except comparison of Age (41–60 yr)-*vs.*-Age (61–80 yr) which was found insignificant ($p > 0.05$). Results reflected that AREG expression has a more sensitive association with the young age (Fig. 1).

AREG is down regulated in patients of various molecular types of BRCA including Luminal (128.99 TPM), HER2 (17.75 TPM) and triple negative breast cancer, *i.e.*, TNBC (20.69 TPM). All comparisons were significant except Normal-*vs.*-Luminal and HER2 Positive-*vs.*-TNBC. TNBC has a worse prognosis due to a set of onco-mutations other than common hormonal-mediated carcinogenesis. AREG was slightly up regulated in TNBC-BL2 (basal-like 2) (128.36 TPM) as compared to TNBC-BL1 (basal-like 1) (29.77 TPM), TNBC- IM (immunomodulatory) (8.78 TPM), TNBC-LAR (luminal androgen receptor) (6.41 TPM), TNBC-M (21.91 TPM) and TNBC-UNS (unstable) (13.26 TPM). Many comparisons were found statistically significant ($p < 0.005$) as shown in Fig. 2 and thus suggest the role of AREG in corresponding TNBC subtypes.

AREG has elevated expression in pre-menopause (227.2 TPM) as compared to peri-menopause (103.92 TPM), post-menopause (64.60 TPM) and normal subjects (164.12 TPM). The comparisons including Normal-*vs.*-Pre-Menopause patients and patient groups (Pre-*vs.*-Peri and Pre-*vs.*-Post-Menopause) were examined to be statistically significant ($p < 0.005$). These results indicate a special connection of AREG expression with menstrual cycle's hormonal pathways. AREG has somehow up regulated expression (Fig. 2) in histological ILC (168.47 TPM) as compared with IDC (92.10 TPM), Mixed (67.72 TPM), other (39.92 TPM), Mucinous (128.22 TPM), Metaplastic (6.42 TPM), IDC-INOS (Invasive breast cancer not otherwise specified) (2.45 TPM) and Medullary (9.28 TPM). Many comparisons among various patient groups (Fig. 2) were noticed to be statistically significant ($p < 0.005$) including one comparison of normal subjects with the patient group (*i.e.*, Normal-*vs.*-Metaplastic).

3.3 Differential expression analysis of AREG protein

The AREG protein expression was slightly higher in primary breast tumors (2.82 *z*-value) as compared to the normal subjects (0.73 *z*-value). AREG protein expression is lower in stage 1 patients (0.56 *z*-value), African-American patients (0.73 *z*-value), 61–80 years patients (0.94 *z*-value), and 81–100 years patients (0.25 *z*-value) than in normal controls (Fig. 3). How-

TABLE 1. AREG pathway level association enrichment analysis.

S. No	Pathway level enrichment
1	MAPK cascade and epidermal growth factor receptor signaling pathway
2	ER to Golgi vesicle-mediated transport
3	G protein-coupled receptor signaling pathway
4	Positive regulation of cell proliferation
5	Response to organic cyclic compound
6	Neuron projection development
7	Negative regulation of osteoblast differentiation
8	Positive regulation of peptidyl-tyrosine phosphorylation
9	Dichotomous subdivision of terminal units involved in mammary gland duct morphogenesis
10	Mammary gland branching involved in thelarche
11	Mammary gland alveolus development
12	Epithelial cell proliferation involved in mammary gland duct elongation
13	Response to cAMP

MAPK: Mitogen-activated protein kinase, ER: Estrogen Receptor, cAMP: Cyclic Adenosine Monophosphate.

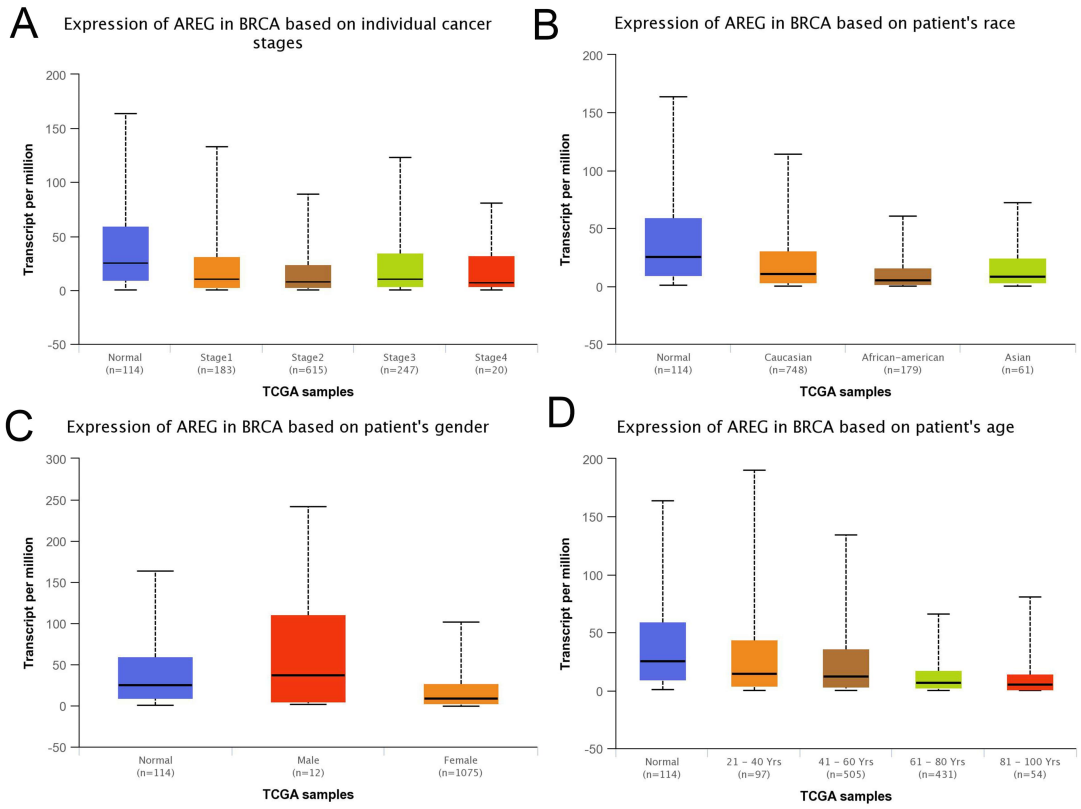

FIGURE 1. Expression of Amphiregulin (AREG) transcripts in breast invasive carcinoma (BRIC) patients of different stages, race, gender and age groups UALCAN database incorporates. TCGA (The Cancer Genome Atlas) consortium based RNA-sequencing data of several normal and cancerous breast tissue patient expression profiles in box-whisker plots. AREG box-whisker plots showed relative expression in both normal breast tissue and breast invasive carcinoma sub-groups. The colored boxes reflected association of expression level (in terms Number of AREG Transcripts per Million, *i.e.*, TPM) with upper quartile range. AREG expression varied heterogeneously among all types of breast cancer tissues (A–D). Statistical analysis revealed significant variations ($p < 0.005$) among following comparisons: (D) Normal-*vs.*-Age (21–40 yr), Normal-*vs.*-Age (81–100 yr), Age (21–40 yr)-*vs.*-Age (41–60 yr), Age (21–40 yr)-*vs.*-Age (61–80 yr), Age (21–40 yr)-*vs.*-Age (81–100 yr), Age (41–60 yr)-*vs.*-Age (81–100 yr) and Age (61–80 yr)-*vs.*-Age (81–100 yr). However, variations among various patients' groups (based upon individual cancer stages (A), patient's race (B) & gender (C)) and patients-*vs.*-Normal group were found to be insignificant ($p > 0.05$). Detail of the statistical significance level is given in **Supplementary Table 5**.

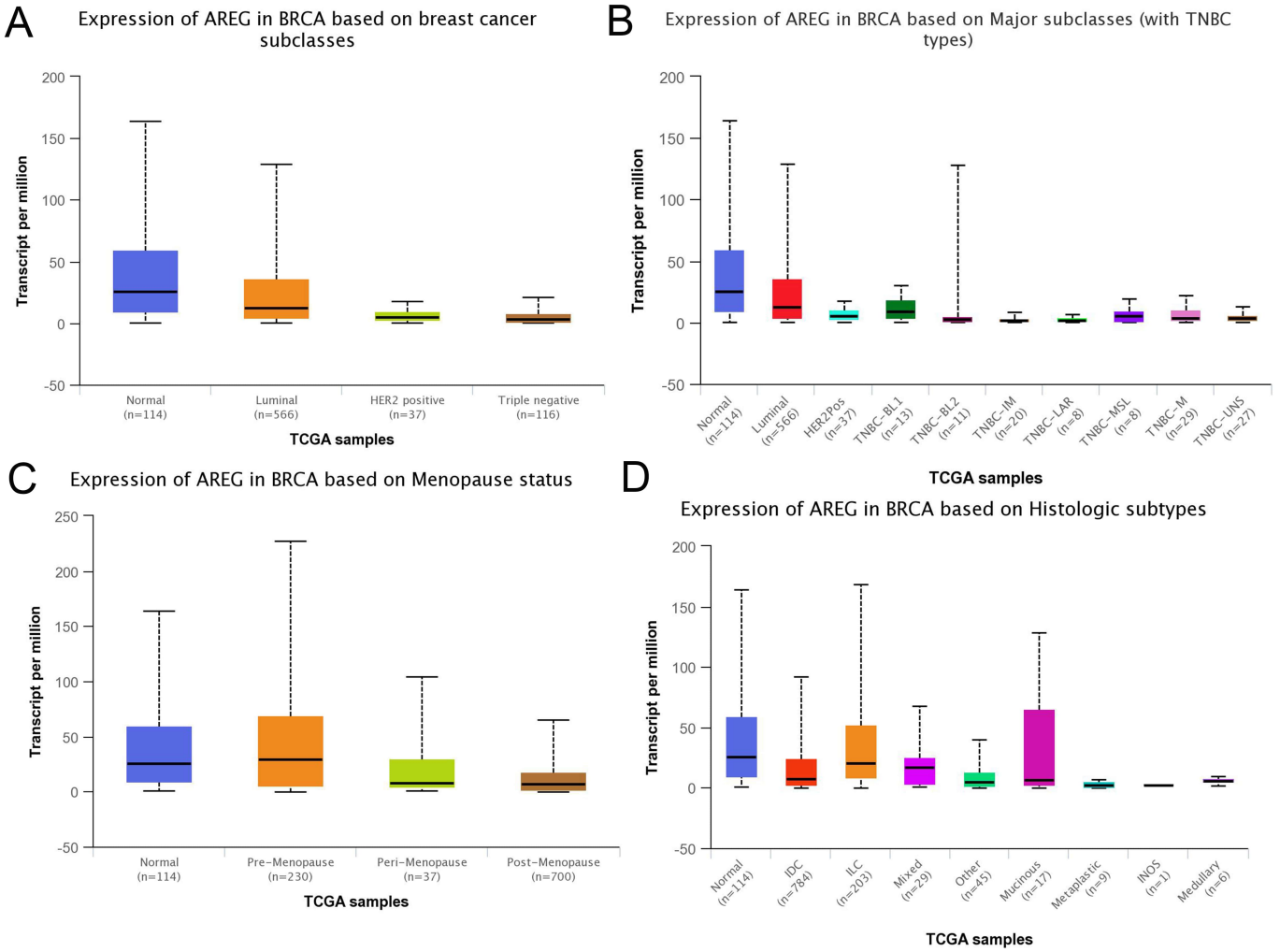

FIGURE 2. Expression of AREG transcripts in Triple Negative Breast Cancer (TNBC) patients of various molecular, histological and menopausal status. The box-whisker plots showed lowered expression (measured in terms Number of AREG Transcripts per Million, *i.e.*, TPM) in many TNBC subtypes, HER2+, IDC (invasive ductal carcinoma), Metaplastic, Medullary and pre-menopause cancer status. However, higher AREG expression (represented as TPM) was observed in ILC (invasive lobular carcinoma) and pre-menopause condition as compared to normal tissue (A–D). Statistical analysis revealed significant variations ($p < 0.005$) among following comparisons: (A) Normal-*vs.*-HER2 Positive, Normal-*vs.*-TNBC, Luminal-*vs.*-HER2 Positive, Luminal-*vs.*-TNBC, (B) Normal-*vs.*-HER2 Pos, Normal-*vs.*-TNBC-BL1, Normal-*vs.*-TNBC-IM, Normal-*vs.*-TNBC-LAR, Normal-*vs.*-TNBC-MSL (mesenchymal stem-like), Normal-*vs.*-TNBC-M, Luminal-*vs.*-HER2 Pos (positive), Luminal-*vs.*-TNBC-BL1, Luminal-*vs.*-TNBC-IM, Luminal-*vs.*-TNBC-LAR, Luminal-*vs.*-TNBC-MSL, Luminal-*vs.*-TNBC-M, Luminal-*vs.*-TNBC-UNS, HER2 Pos (positive)-*vs.*-TNBC-LAR, TNBC-BL1-*vs.*-TNBC-LAR, TNBC-LAR-*vs.*-TNBC-M, (C) Normal-*vs.*-Pre-Menopause, Pre-Menopause-*vs.*-Peri-Menopause, Pre-Menopause-*vs.*-Post-Menopause, (D) Normal-*vs.*-Metaplastic, IDC-*vs.*-Metaplastic, ILC-*vs.*-Other, ILC-*vs.*-Metaplastic, Mixed-*vs.*-Metaplastic, Other-*vs.*-Metaplastic and Mucinous-*vs.*-Metaplastic. However, variations among various patients' groups (based upon breast cancer subclasses, major subclasses (with TNBC types), menopause Status & histologic Subtypes) and patients-*vs.*-Normal group were found to be insignificant ($p > 0.05$). Detail of the statistical significance level is given in **Supplementary Table 6**.

ever, higher protein expression was documented in stage 2 (1.48 *z*-value), stage 3 (2.59 *z*-value), Caucasian (2.47 *z*-value), Asian (1.07 *z*-value), 21–40 years (2.73 *z*-value) and 41–60 years (1.47 *z*-value). But statistical analysis revealed all variations were statistically insignificant ($p > 0.05$). Hence, variations in the expression levels of AREG protein may not be correlated with breast cancer subclasses, and the patients' gender, race and age. Moreover, we cannot generalize these findings as conclusive measures of BRCA patients because of the low number of samples in protein expression datasets.

AREG protein expression is higher than normal in Luminal (1.48 *z*-value) and IDC (2.28 *z*-value) while lower protein expression in HER2+ (0.35 *z*-value), TNBC (0.94 *z*-value), ILC (0.75 *z*-value) and Mixed breast cancer types (0.52 *z*-value). However, only HER2+ BRIC patients differed significantly ($p < 0.05$) from normal subjects and luminal BRIC patients (Fig. 4).

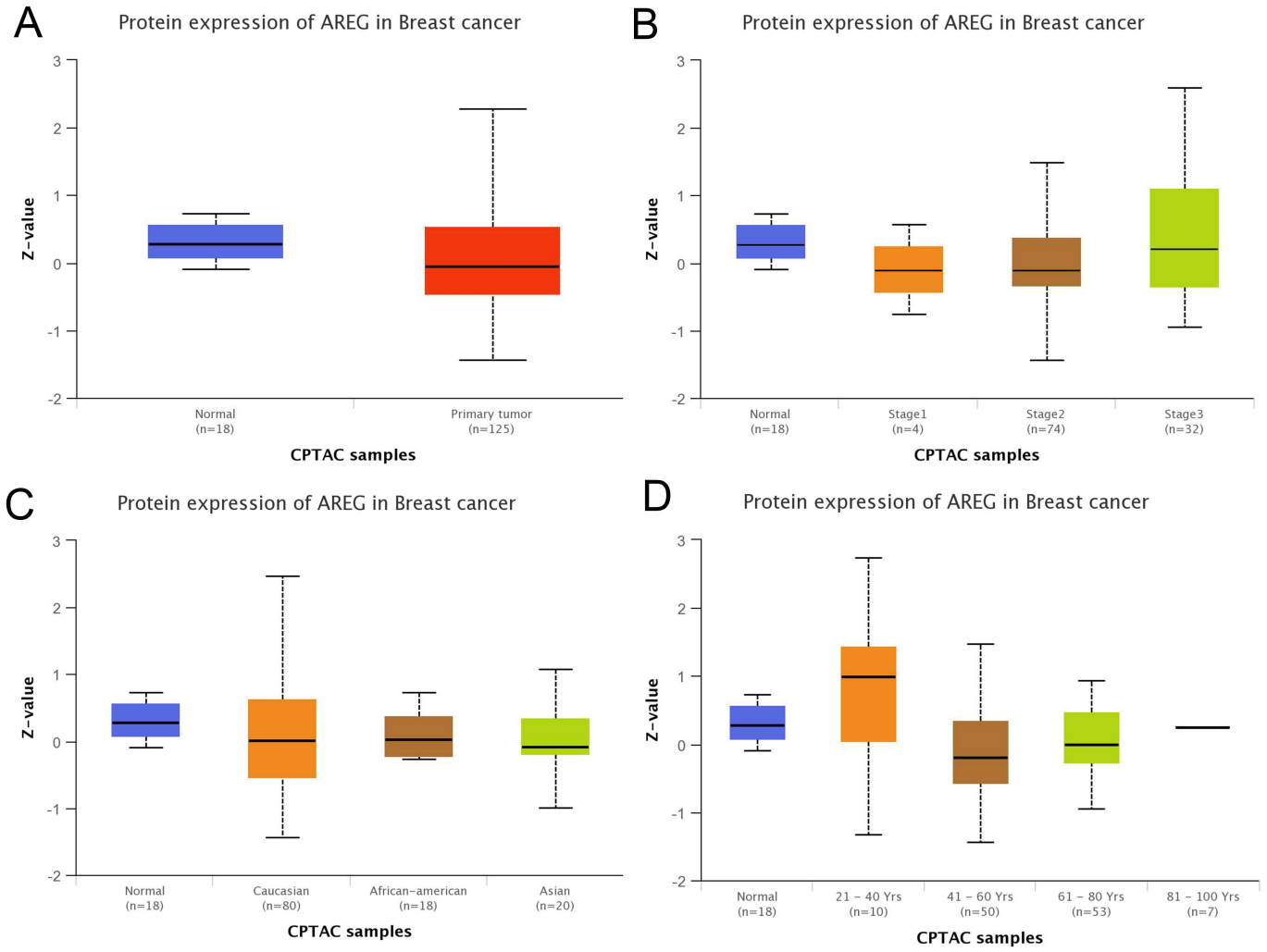

FIGURE 3. Expression of AREG (Amphiregulin) protein in BRIC (Breast Invasive Carcinoma) patients of primary tumor, different stages, race, gender and age groups. *z*-value indicates standard deviations from the median documented across samples of a particular group. The log2 values of spectral count ratio, obtained from CPTAC (Clinical Proteomic Tumor Analysis Consortium, were first normalized within profile of each type/group of sample profile followed by normalization across the samples. The number of samples in a particular dataset is represented by n. Statistical analysis revealed non-significant variations ($p > 0.05$) among all comparisons shown in the (A–D). Higher expression of AREG was detected in primary tumor (A), stage 2, stage 3 (B), Caucasian, Asian (C), 21–40 years and 41–60 years (D) patients. While in all other comparisons expression was lowered than reference but the variation was insignificant (A–D). Detail of the statistical significance level is given in **Supplementary Table 7**.

3.4 AREG differential promoter methylation analysis

In this, *in silico* survey, AREG showed hyper-methylation in stage 1 (0.81 β-value), stage 2 (0.83 β-value), stage 3 (0.76 β-value), stage 4 (0.68 β-value), Caucasian (0.83 β-value), African-American (0.81 β-value), Asian (0.72 β-value), male (0.68 β-value), female (0.83 β-value), 21–40 years (0.75 β-value), 41–60 years (0.80 β-value), 61–80 years (0.83 β-value), 81–100 years (0.63 β-value) as compared to normal methylation (0.48 β-value). This hyper-methylation status seems to be a significant factor that reduces AREG expression in tumor cells (Fig. 5). Although the patients exhibited hyper-methylation as compared to the normal subjects only two comparisons (like Normal-*vs.*-Stage2 and Normal-*vs.*-Stage3) in the case of stage-specific groups, all comparisons except Normal-*vs.*-Caucasian and African-American-*vs.*-Asian among race-specific groups, comparison of Normal-*vs.*-Female and comparisons of few age-specific groups Normal-*vs.*-Age (21–40 yr), Normal-*vs.*-Age (41–60 yr) and Normal-*vs.*-Age (61–80 yr) were found to be statistically significant ($p < 0.05$).

3.5 AREG mutatome map extraction

We analyzed the status of AREG regulation in the Breast Invasive Carcinoma TCGA Cell 2015 dataset that helped us to detect amplification in 15 samples and just a single missense mutation in 1 sample. These results strengthened the conclusions of previous studies and highlighted the possibility of AREG acting as a factor strongly promoting breast tissue transformation and growth. AREG has 252 Amino Acids (AA)

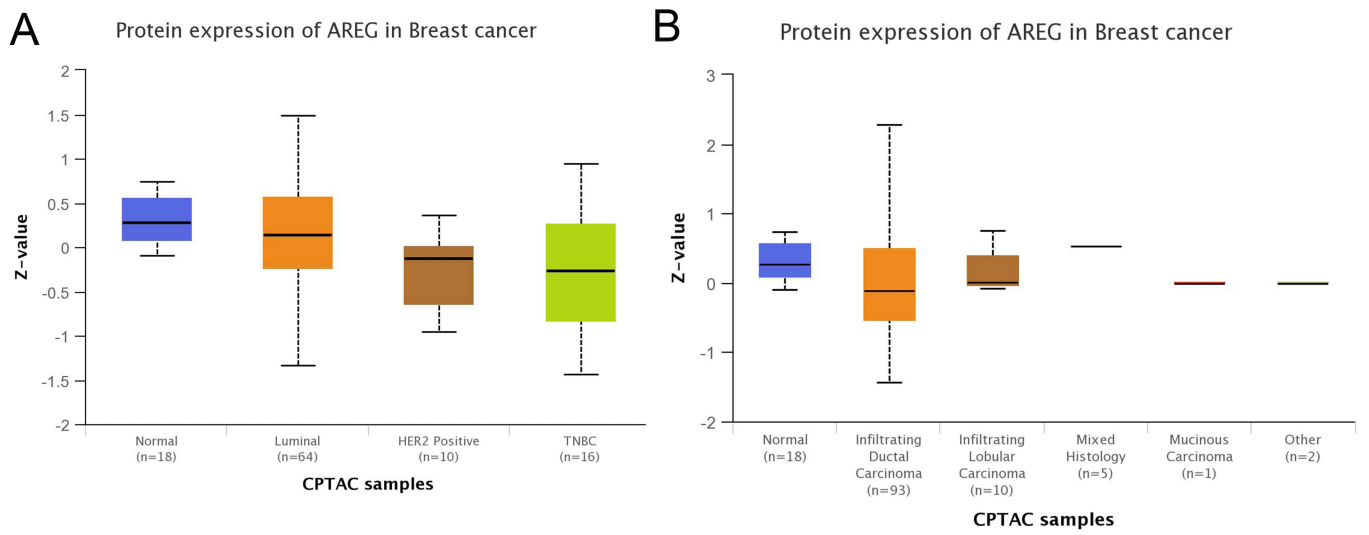

FIGURE 4. Expression of AREG (Amphiregulin) protein in BRIC (Breast Invasive Carcinoma) cancer subclasses and histological types. *z*-value indicates standard deviations from the median documented across samples of a particular group. The log2 values of spectral count ratio, obtained from CPTAC (Clinical Proteomic Tumor Analysis Consortium), were first normalized within profile of each type/group of sample profile followed by normalization across the samples. The number of samples in a particular dataset is represented by n. Statistical analysis revealed significant variations ($p < 0.05$) among following comparisons: Normal-*vs.*-HER2 Positive and Luminal-*vs.*-HER2 Positive (A). However, variations among various patients' groups (based upon breast cancer subclasses (A) and histological types (B)) were found to be insignificant ($p > 0.05$). Detail of the statistical significance level is given in **Supplementary Table 8**.

and among these only a single missense mutation S14W has been observed that has unknown significance (Fig. 6).

3.6 AREG drug sensitivity analysis

AREG is capable to enhance the effectiveness of various anti-cancer drugs (Table 1) including inhibitor of tyrosine kinase receptors (Erlotinib), an inhibitor of VEGF (vascular endothelial growth factor) receptors (Vandetanib), Saracatinib (an inhibitor of Src/Abl kinases) which facilitates rapid invasive proliferation (AZD0530), an inhibitor of MAPK (Mitogen-activated protein kinase)-ERK (extracellular-signal-regulated kinase)-MEK (MAPK/ERK kinase) pathways (PD-0325901) and antineoplastic histone deacetylase inhibitor (Panobinostat). AREG strongly resists the effectiveness of anti-amyloid beta precursor protein that is known to be a promoter of the Alzheimer disease (L-685468) (Table 2).

3.7 Effect of AREG transcripts expression on survival of BRIC patients

Approximately 50% of the patients having a higher or lower/medium expression levels of AREG transcripts have equal survival rates (*i.e.*, about 4500 days). Although a small fraction of the patients having the low/medium expression exhibited the possibility to survive for more days, the overall difference between the two groups was found to be statistically insignificant ($p = 0.096$). When the combined effect of AREG's expression and gender on the patients' survival was evaluated results revealed that few female patients having lower/medium expression levels may possibly survive for more days than the average survival of 4800 days ($p = 0.28$) documented in the case of a majority

TABLE 2. Correlational analysis of AREG expression and drug sensitivity in BRIC.

S. No	Drug	Targets	Correlation
1	Erlotinib	EGFR	Moderate negative
2	Vandetanib	ABL and EGFR	Moderate negative
3	L-685468	Gamma secretase	Strong positive
4	AZD0530	Src, ABL and BCR	Moderate negative
5	PD-0325901	MEK	Moderate negative
6	Panobinostat	HDAC	Moderate negative

AREG expression is associated ($p = 0.0498$) with drug sensitivity in BRIC tissues. Positive correlation means that the drug is less effective and a negative correlation reflects enhanced effectiveness. AREG expression confers drug sensitivity to BRIC tissues. While negative correlation means AREG expression enhances resistivity against particular drug. The strength of correlation is ranked over 03 degrees, i.e., low, moderate & strong. AREG expression showed strong resistive role against gamma secretase inhibitor. Epidermal Growth Factor Receptor (EGFR), Mitogen-activated protein kinase (MEK), Abelson tyrosine protein kinase (ABL), Histone deacetylase (HDAC) and Breakpoint cluster region protein (BCR).

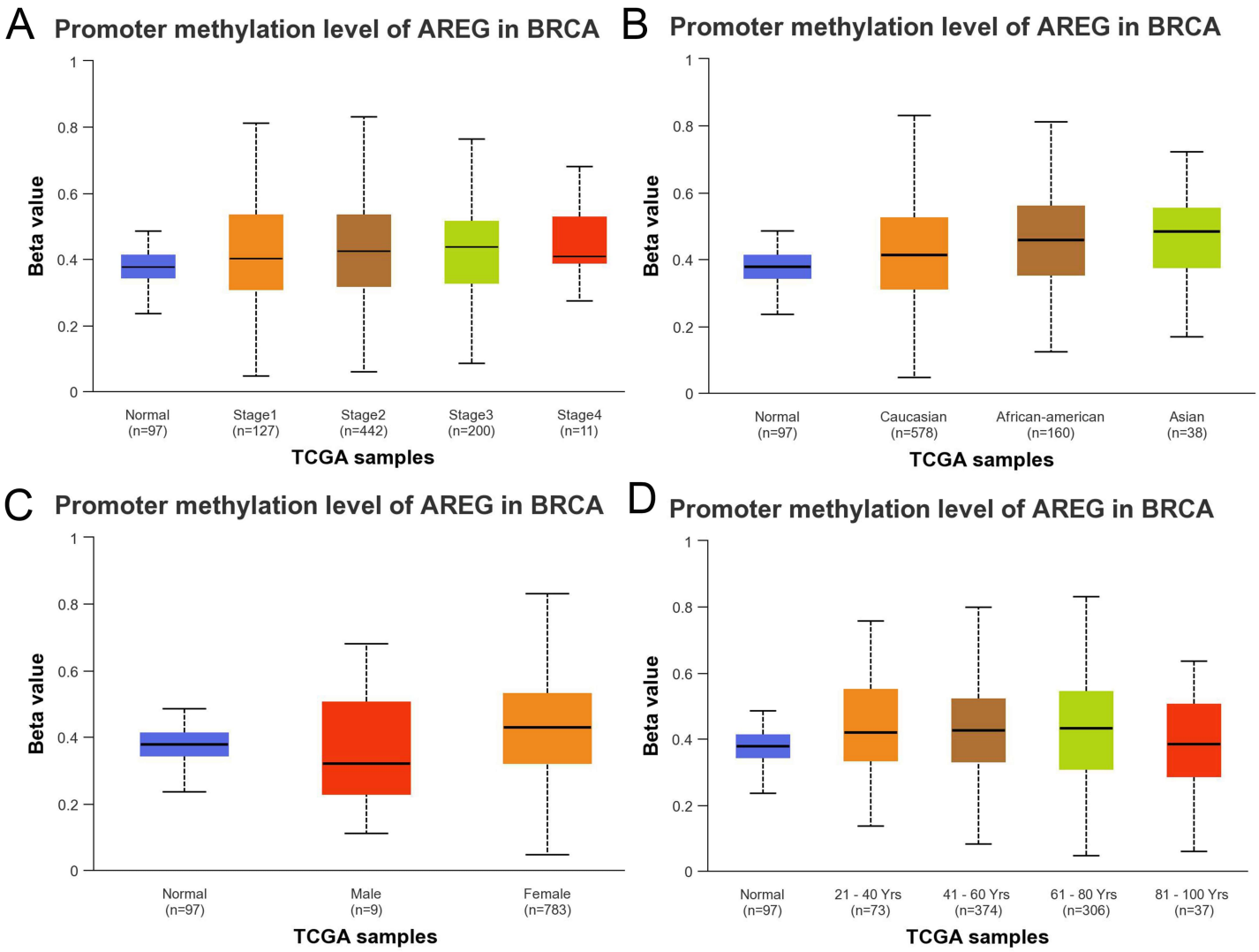

FIGURE 5. Promoter methylation status of AREG (Amphiregulin) among various sub-groups of Breast Cancer (BRCA). AREG box-whisker plots showed promoter methylation expression in normal subjects and various groups of patients categorized on the basis of attributes like stage of disease, race, gender and age. The promoter methylation status is ranked through beta-value [22]. Results showed hyper methylation of AREG promoter in all tumorigenic scenarios as compared with normal tissues (A–D). Statistical analysis revealed significant variations ($p < 0.005$) among following comparisons: Normal-*vs*.-Stage2, Normal-*vs*.-Stage3 (A), Normal-*vs*.-African American, Normal-*vs*.-Asian, Caucasian-*vs*.-African American, Caucasian-*vs*.-Asian (B), Normal-*vs*.-Female (C), and Normal-*vs*.-Age (21–40 yr), Normal-*vs*.-Age (41–60 yr) and Normal-*vs*.-Age (61–80 yr) as shown in (D). However, variations among methylation status of various patients' groups and patients-*vs*.-Normal group were found to be insignificant ($p > 0.05$). Detail of the statistical significance level is given in **Supplementary Table 9**.

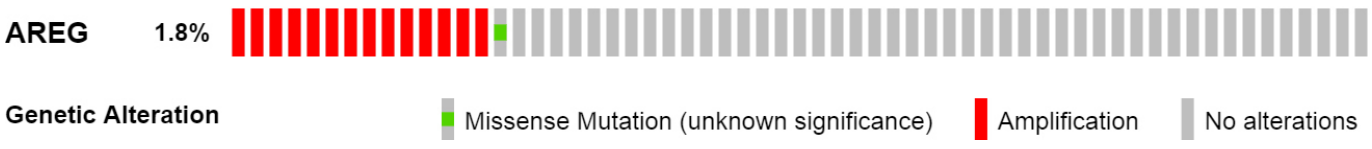

FIGURE 6. An overview of genetic alterations contributing towards AREG (Amphiregulin) copy number regulation. This information was retrieved from datasets available in TCGA (the cancer genome atlas) Cell 2015 BRIC dataset. The vertical bars showed patient sample's data. While the coloring pattern represents type of genetic alteration like amplification and deletion or missense mutation. Majority of the patients showed AREG amplification along with a single case (TCGA-AR-A24Q, female, 49 years old, IDC and white race) of missense mutation. AREG: Amphiregulin.

of patients belonging to both groups. Similarly effect of AREG expression level and patient's race was observed to insignificant on the overall survival duration of patients (p = 0.16). The combined effect of AREG expression and cancer type significantly ($p < 0.001$) contributed to altering the overall survival duration of patients. Luminal BRCA patients having lower/medium AREG expression levels tend to survive more than luminal BRCA patients having higher expression levels and patients of all other groups (Fig. 7). Triple −ive BRCA patients who have low/medium levels of AREG's expression tend to have more chances of survival than the patients of same group but exhibiting higher expression of AREG. The same pattern was observed in HER +ive patients. KM analysis revealed a higher possibility of survival in the case of post-menopausal patients having lower/medium levels of AREG's expression than corresponding patients having a higher AREG's expression (p = 0.03). It was further noticed that the post-menopausal patients harboring higher or lower/medium levels of AREG's expression have more chance to survive than the peri & pre-menopausal patients.

4. Discussion

Breast cancer recurrence, worse prognosis and deaths are strongly associated with EGFR overexpression. In literature minimal success stories are known regarding anti-EGFR therapies in BRIC [25–27]. In ER-positive breast tumors AREG is observed to be overexpressed and its loss of function reduces the invasiveness capabilities of carcinoma. The estrogen receptor signaling pathway regulates the expression level of AREG which is essential for mammary glands' development during puberty and AREG's dysfunction leads to the formation of abnormal ductal branching system in breast [28–30]. Epithelial EGFR/AREG levels are sensitive markers in normal mammary tissue which keep on building throughout childhood [31]. The estrogen related signaling pathway up regulates AREG/EGFR's expression and thus facilitates the development of milk-carrying ducts [32, 33]. The fibroblasts growth factors (FGF) which is regulated by AREG stimulates branching of the breast ducts by bind with FGF receptors present on the epithelial cells [34].

In the present study, AREG exhibited a complex expression pattern in BRIC datasets. The patients of different BRCA stages showed lowered expression of AREG transcripts than observed in the case of normal subjects. Although the difference is statistically insignificant ($p > 0.05$) and stage 1 patients had slightly higher expression of transcripts as compared to patients of advanced stages. As the tumor proceeds to higher levels AREG's expression is further lowered. Our results suggest that AREG's expression levels may help to discriminate between primary and advanced-grade tumors. AREG's up regulation invites to design of combinatorial therapy against EGFR/AREG cascades to prevent rapid cell proliferation. AREG's expression is slightly higher in the Caucasian population which is a combination of Asian, African and European races but insignificantly ($p > 0.05$) varied from the normal group and other races based patient groups. AREG's expression is higher in male patients than the normal expression and female patients. This observation opens a new avenue of gender-based differential expression of AREG in carcinogenesis. AREG's expression is also significantly higher ($p < 0.05$) than normal in 21–40 and 81–100 years old cases. Moreover, many age-based groups of patients varied significantly from each other ($p < 0.05$). Hence, it is concluded that AREG's expression may be utilized as a remarkable prognostic marker for a particular age group with consideration of age-specific variations. AREG's expression is higher in hormone receptors positive luminal carcinomas than triple-negative breast carcinomas ($p < 0.05$) which showed a correlation of AREG's and hormone receptors' expression. AREG has significant overexpression in premenopausal cases than normal expression ($p < 0.05$) which indicates its strong association with estrogen cycle pathway. AREG's expression in patients suffering from various histologic subtypes of BC exhibited significant variation ($p > 0.05$) than normal expression (Fig. 2). AREG association with various histologic subtypes of BRCA depicts the impact of hormone-based pathways, particularly the one which is induced by epidermal growth factors, on tumorigenesis and cell proliferation.

CPTAC analysis revealed significant variations in the case of HER2+ BRIC patients. The transcriptional level of expression correlates with the expression levels of AREG protein in HER2+ patients. All other comparisons were found to be statistically insignificant ($p > 0.05$). Hence, AREG protein's expression data did not help to get a conclusive overview. This might be due to a limited number of samples in each data set.

Our study indicated that lowered expression of AREG transcripts in BRIC patients than its expression in normal subjects is due to promoter's hyper-methylation. AREG hypermethylation demands thorough investigation to investigate the differential behavior of methyl transferases and associated pathways in normal to tumorigenic situations. Which are molecular inducers of such methylation pathways? Will it be possible in the future to modulate the activity of methylation pathways by selective epigenomic engineering to turn on the tumor suppressor genes and turn off the oncogenes? It was noticed that AREG's expression enhances the effectiveness of anti-neoplastic drugs. However, the strong resistive role against gamma-secretase inhibitors reflects the role of AREG in drug resistance pathways. KM survival curve analysis revealed that lower expression level of AREG promotes the survival of patient in luminal, triple −ive and post-menopausal patients. However, apparently there is no impact of AREG's expression on a patient's survival in race and gender specific patients' groups.

Our findings suggest the use of AREG's expression as a prognostic marker to predict the survival of BRIC patients. This study provokes researchers to further address the impact of AREG down regulation on balancing proliferation rate in either normal breast or tumor cases. There is a need of further studies to explore the influence of AREG's differential expression and its promoter's methylation in BRIC.

5. Conclusions

The majority of the available data was based upon the transcriptional expression level of AREG. Hence, most of the conclusions of our study are based upon the expression sta-

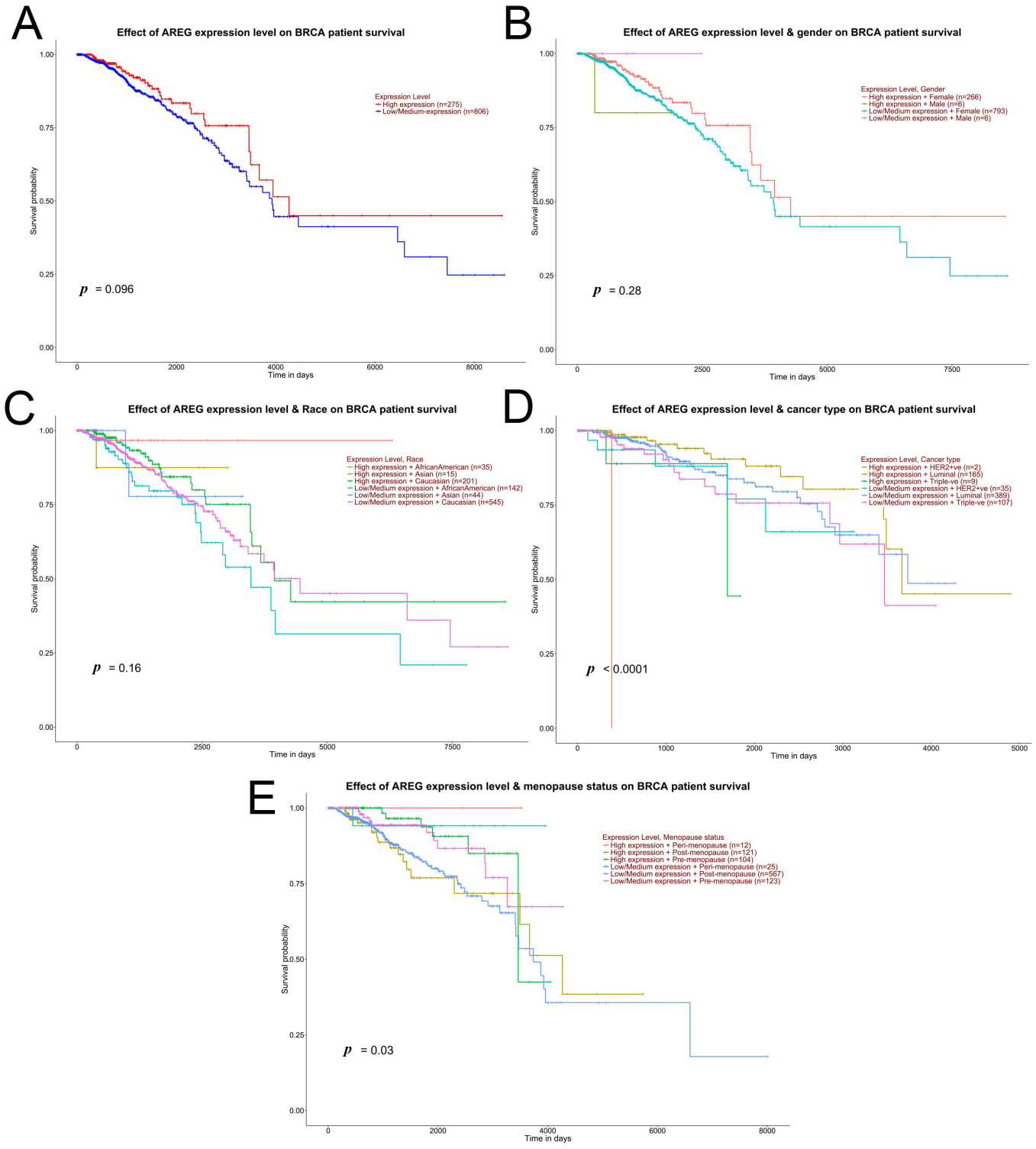

FIGURE 7. Correlation AREG (Amphiregulin) expression with survival of BRIC (Breast Invasive Carcinoma) patient data either with general expression level or Patient's race, gender and cancer subtypes. This figure has summarized information regarding effect of various factors on the survival of breast cancer patients. Data is presented in the form of KM (Kaplan–Meier) plots (A–E). The chance of survival seems to be higher ($p > 0.05$) in the majority of patients having the low/medium expression than patients having a higher or lower/medium expression levels of AREG transcripts (A) and few female patients having lower/medium expression levels (B). The effect of AREG expression level and patient's race was insignificant ($p = 0.16$) on the overall survival duration of patients (C). However, the breast cancer subtypes have significant impact on patients' survival. The survival chance was higher in Luminal BRCA patients, Triple −ive BRCA patients, and HER +ive patients having lower/medium AREG expression levels than the other patients of their corresponding groups (D). Similarly post-menopausal patients, having lower/medium levels of AREG's expression, seem to have higher survival chance ($p = 0.03$) than other post-menopausal patients and peri and pre-menopausal patients (E).

tus of AREG transcripts unless otherwise mentioned. Our study revealed the unique role of AREG transcripts in multiple BRIC-associated variables. Our results suggested the role of DNA methylation machinery in down regulation of AREG, the influence of hormone-related pathways in AREG' up regulation, and participation of AREG in drug sensitivity or resistance pathways, and highlighted the potential of AREG's expression to predict the overall survival of BRIC patients. The information thus generated may trigger researchers worldwide to design anti-tumor drugs targeting AREG and kinases and playing a role in regulating AREG-related methylation pathways. Our findings demand extensive efforts to further explore the mechanistic details regarding the role of AREG's expression in the pathogenesis of BRIC, drug resistance pathways, and survival of BRIC patients prior to the clinical implication of AREG as a prognostic marker of BRIC.

AVAILABILITY OF DATA AND MATERIALS

All related data is contained within this article and supplementary material.

AUTHOR CONTRIBUTIONS

ZAS, FN and SE—conceived and designed this study. ZAS—carried out all bioinformatics analysis and wrote first draft of paper. All authors contributed to finalize the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not Applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found, in the online version, at https://oss.ejgo.net/files/article/1846066156892241920/attachment/Supplementary%20material.docx.

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