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High expression of DLG3 is associated with decreased survival from breast cancer

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Abstract

Abnormal expression or activity of proteins that regulate cell polarity can contribute to tumour progression. Discs large homolog (DLG) proteins play crucial roles in the maintenance of cell polarity and tissue morphogenesis. Previous studies of breast cancer patients showed that DLG3 had greater expression in the cancerous tissues than non-cancerous tissues, but the relationship between DLG3 expression and breast cancer progression and prognosis is not clear. Here, we investigated the association of DLG3 expression with breast cancer progression and prognosis using data on clinicopathological parameters from The Cancer Genome Atlas (TCGA) database, with different clinicopathological parameters using UALCAN and LINKEDOMICS, and with different stages and subtypes using immunohistochemical staining. The results indicated greater DLG3 expression in cancerous breast tissues than normal breast tissues and in luminal and Her2+ subtypes than in the triple-negative subtype. DLG3 expression also had a positive correlation with pathologic stage and decreased survival rate. Our data suggest that DLG3 should be considered as a new diagnostic and prognostic biomarker for breast cancer.

KEYWORDS
breast cancer, diagnosis, DLG3, prognosis

1 | INTRODUCTION

Worldwide estimates have indicated that breast cancer was the most commonly diagnosed cancer (11.6% of 18.1 million new cases) and the fifth leading cause of cancer mortality (6.6% of 9.6 million deaths) during 2018.[1,2] Breast cancer is also the most common cancer and the leading cause of cancer deaths among women worldwide. In North America and the European Union, the mortality rate from breast cancer has decreased during recent years due to improvements in early detection and systemic therapies. However, in less developed countries, breast cancer still has the highest mortality rate among all cancers. Lifestyle changes, lack of early detection, and fewer screening programs are the main reasons for the continuing high incidence and mortality of breast cancer in South America, Africa, and Asia. Because early-stage breast cancer is potentially curable,[3] identification of new biomarkers for diagnosis or to guide therapy may reduce mortality rates.

Most human cancers originate from epithelial cells, and polarity proteins play crucial roles in maintaining epithelial structure and function.[4] Cell adhesion and cell polarity complexes (including the crumbs [CRB] complex, partition defective [PAR] complex, and scribble [SCRIB] complex) play key roles in maintaining epithelial cell

polarity. In mammals, the CRB complex consists of CRB1-3, a protein associated with Lin seven 1 (PalS1), and PALS1-associated tight junction protein (PATJ). The PAR complex consists of PAR3 and 6, and atypical protein kinase C (aPKC). The SCRIB complex consists of scribble, lethal giant larvae (LGL), and discs large (DLG). Abnormal expression of cell adhesion and cell polarity proteins is associated with tumour progression and invasiveness.[5,6] Recent research indicated alterations in the expression of many cell polarity proteins in human cancers.[7,8] For example, there is increased expression of PAR6 and aPKC in breast cancer, non-small cell lung cancer, and ovarian cancer.[5] In contrast, CRB3, PAR3, LGL2, DLG1, and DLG5 function as tumour suppressors in many cancers.[9-13]

Discs large homolog 3 (DLG3), also known as SAP102 (synapse-associated protein 102), is a mammalian homolog of *Drosophila* DLG tumour suppressor. DLG3 is also in the membrane-associated guanylate kinase (MAGUK) superfamily, whose members contain several PDZ (PSD-95/DLG/ZO-1) domains, an src homology 3 (SH3) domain, and a region with high similarity to guanylate kinases (GK).[14-16] MAGUK proteins regulate epithelial cell polarity, synaptic development, and synaptic plasticity.[17,18] Directed trafficking of DLG3 plays important roles in different polarized cell types and in the establishment and maintenance of apical cell junctions and tight junctions of epithelial cells and neuronal synapses.[19,20] The level of DLG3 mRNA is greater in cancerous breast, kidney, liver, lung, and ovarian tissues than in normal tissues.[21]

In this study, we first analyzed the association of DLG3 expression with the pathological features and probability of survival from breast cancer in The Cancer Genome Atlas (TCGA) database using the UALCAN and LINKEDOMICS web tools. Next, we measured DLG3 expression in breast cancer tissues by immunohistochemical staining and assessed its use as a diagnostic and prognostic biomarker for breast cancer.

2 | RESULTS

2.1 | Association of DLG3 expression with breast cancer

We initially used the UALCAN web tool (http://ualcan.path.uab.edu/index.html) to compare DLG3 mRNA expression in adjacent normal
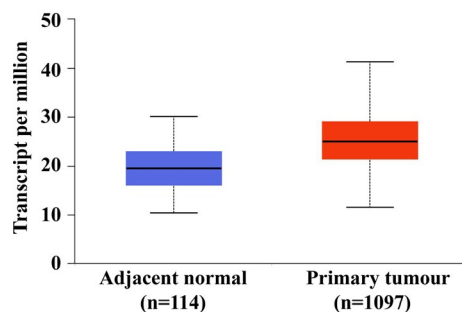
FIGURE 1 Expression of DLG3 mRNA in adjacent normal tissue and breast cancer tissues ($P = 1.62 \times 10^{-12}$). Box plots were produced by UALCAN (http://ualcan.path.uab.edu/index.html)

Impact statement

Worldwide, breast cancer is the most common cancer and the leading cause of cancer deaths among women. Identification of new biomarkers for diagnosis, therapy and prognosis may reduce mortality rates of breast cancer. DLG3 was highly expressed in the breast, kidney, liver, lung, and ovary cancers. The major finding of our study is higher DLG3 expression in cancerous breast tissues than normal breast tissues. We also found that high expression of DLG3 was associated with poor prognosis. Our findings may shed a new light on DLG3 which might be useful as a diagnostic and prognostic indicator for breast cancer.

tissue (n = 114) and cancerous breast tissue (n = 1097) specimens. The results indicate that DLG3 expression was significantly greater in breast cancer tissues ($P = 1.62 \times 10^{-12}$) (Figure 1).

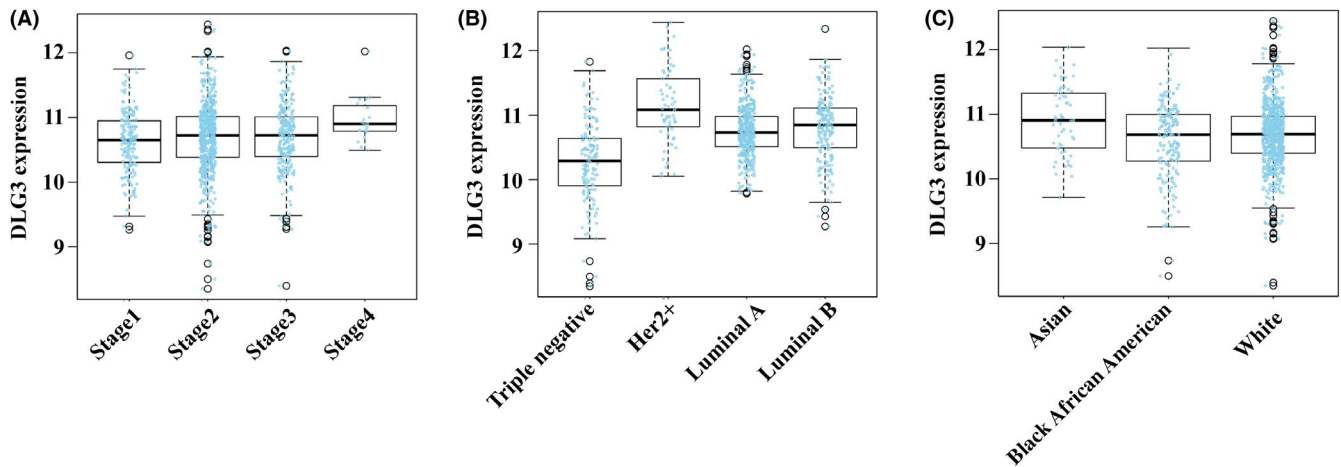### 2.2 | Association of DLG3 expression with pathological features of breast cancer

Next, we analyzed the association of DLG3 expression with the pathological features of breast cancer using the LINKEDOMICS web tool (http://www.linkedomics.org/login.php). The results indicate that DLG3 correlated with pathologic stage (n = 1071, $P = 1.19 \times 10^{-2}$), with cancer subtype (n = 826, $P = 1.33 \times 10^{-25}$), and with race/ethnicity (n = 997, $P = 4.88 \times 10^{-3}$; Figure 2). Further analysis of Asian patients indicated DLG3 expression was greater in those with more advanced pathologic stage and with the luminal A/B and Her2+ subtypes.

2.3 | Association of DLG3 expression with survival

Our analysis of the TCGA database using the UALCAN web tool indicated there was a significant association of DLG3 expression with the overall mortality of breast cancer patients (Figure 3A, $P = 3.40 \times 10^{-4}$). In addition, separate analysis of women with three different breast cancer subtypes (luminal A/B, Her2+, and triple-negative) indicated high DLG3 expression was associated with reduced survival time in all three groups (Figure 3B, $P = 6.70 \times 10^{-3}$).

2.4 | IHC of DLG3 expression

We next used IHC staining to confirm the association of DLG3 expression with pathological features of breast cancer from the TCGA datasets (Figure 4, Table 1). The results indicated that DLG3 expression was highest in stage 3 cancer tissues, and lowest in stage 1 cancer tissues (Figure 4A,C). Further analysis of breast cancer subtypes indicated DLG3 expression was greater in the luminal A/B and Her2+ subtypes than in the triple-negative subtype (Figure 4B,D). Both in the luminal A/B or Her2+ subtypes and in the triple-negative

FIGURE 2 Association of DLG3 expression with (A) breast cancer pathological stage (n = 1071, $P = 1.19 \times 10^{-2}$, Kruskal–Wallis test), (B) cancer subtype (n = 826, $P = 1.33 \times 10^{-25}$, Kruskal–Wallis test), and (C) race/ethnicity (n = 997, $P = 4.88 \times 10^{-3}$, Kruskal–Wallis test). Box plots were produced using LINKEDOMICS (http://www.linkedomics.org/login.php)

subtype, there is a positive correlation of DLG3 expression with pathological stage (Figure 4E,F).

## 2.5 | Association of DLG3 expression with survival

We also examined the association of DLG3 expression with survival rate by extraction of survival data of patients whose tissue microarrays were examined. The results of the univariate analysis, using Kaplan-Meier analysis and the log-rank test, indicated that high DLG3 expression was significantly associated with shorter overall survival (OS; Figure 5A, $P = 1.20 \times 10^{-2}$). Separate analysis of the luminal A/B and Her2+ subtypes also indicated high DLG3 expression was associated with reduced OS (Figure 5B, $P = 1.50 \times 10^{-2}$). There was no such association for the triple-negative subtype ($P = .29$, Figure 5C), although we only analyzed data for 29 triple-negative patients. Taken together, the results from our analysis of the TCGA database and tissue microarrays indicate that the level of DLG3 expression correlates with the presence of breast cancer, with more advanced cancer, and with poor OS from breast cancer.

3 | DISCUSSION

Breast cancer is the most common and most lethal of cancers among women worldwide. New tools for the diagnosis and prediction of prognosis may enable earlier onset of treatment and reduce mortality from breast cancer. Many cell polarity proteins have known roles in breast cancer and several other malignancies.[5] Previous research indicated that the levels of DLG3 (a cell polarity protein also named SAP102) were higher in cancers of the breast, kidney, liver, lung, and ovary.[21] Here, we found higher DLG3 expression in cancerous breast tissues than healthy breast tissues and in luminal A/B and Her2+ subtypes than in the triple-negative subtype by using data on clinicopathological parameters from the TCGA database. We also found that high expression of DLG3 was associated with poor prognosis. There were several
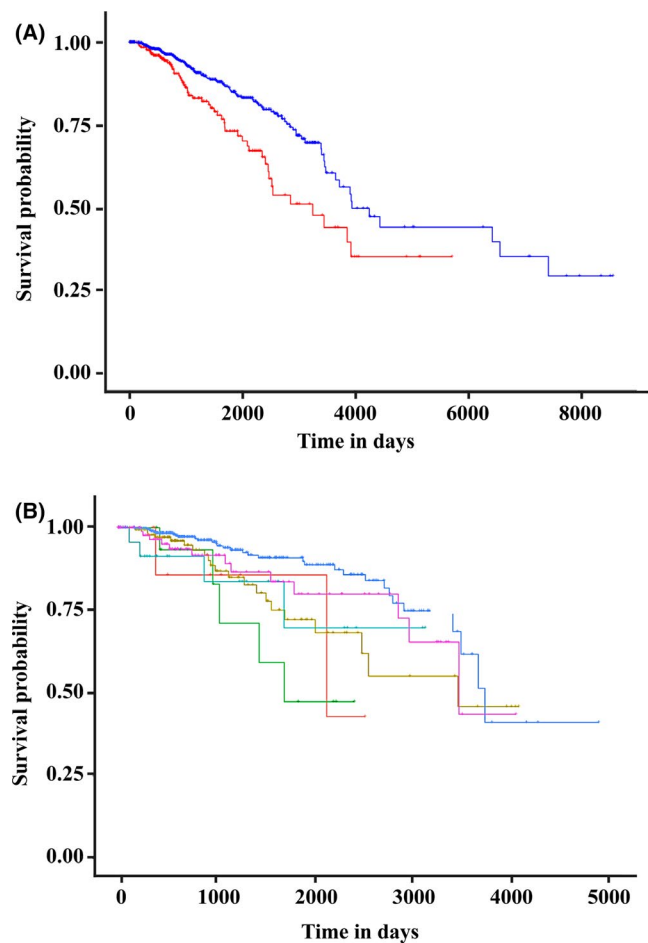
FIGURE 3 Association of DLG3 expression with OS of (A) breast cancer patients ($P = 3.40 \times 10^{-4}$) and (B) with OS of patients with different breast cancer subtypes ($P = 6.70 \times 10^{-3}$). Kaplan-Meier curves were produced using UALCAN (http://ualcan.path.uab.edu/index.html). A, Expression level: High expression (n = 271); Low/medium expression n = 810); B, Expression level, cancer type: (Luminal: High (n = 146); Low (n = 408); Her2+ High (n = 11); Low (n = 26); Triple negative High (n = 16); Low (n = 100)
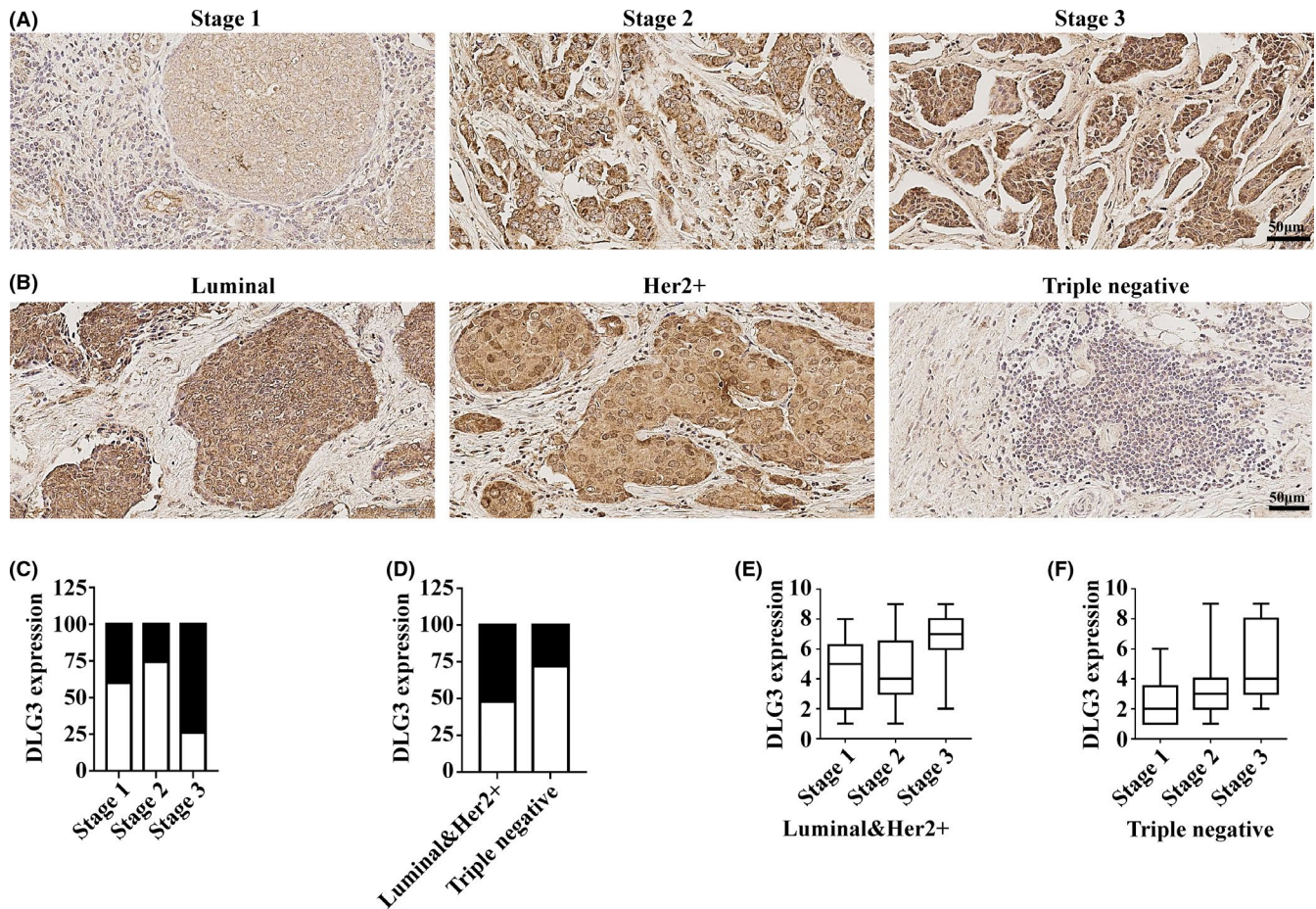
FIGURE 4 Immunohistochemical analysis of DLG3 expression in representative breast cancer tissue samples of different pathologic stages (A, C; $P < .0001$, one-way ANOVA) and different subtypes (B, D; $P = 3.00 \times 10^{-4}$, un-paired t test). Bar = 50 μm. The DLG3 expression of different staging within the luminal A/B or Her2+ subtypes (E; $P < .0001$, one-way ANOVA) or in triple-negative subtypes (F; $P = 1.06 \times 10^{-2,}$ one-way ANOVA) C, ■ High; □ Low; D, ■ High; □ Low

limitations of this study. First, because this study had a retrospective design, we can only identify associations, and cannot infer casualties. Second, there was no correction for confounding, so any reported association may be due to an unknown intervening factor(s). Nonetheless, we confirmed an association of DLG3 expression with the pathological features of breast cancer by IHC staining of breast cancer tissue microarrays and according to analysis of the TCGA database.

Many proteins in the MAGUK superfamily function in the maintenance of epithelial polarity and cell junctions, and abnormal expression of these proteins is associated with tumour progression. For example, DLG1 has decreased expression in some cervical and breast cancers,[22,23] a new isoform of DLG2 has increased expression in renal oncocytoma,[24] and ZO1 has decreased expression in breast cancer.[25] The present study suggests that DLG3 may function as an oncogene in breast cancer, although it also appears to act as a tumour suppressor in papillary thyroid carcinoma and glioblastoma.[26] The mammalian DLG3 protein is structurally similar to the *Drosophila* protein DLG A, which functions as a tumour suppressor in many types of cancers.[16] DLG3 overexpression induces mitotic cell cycle arrest and apoptosis, and inhibits cell proliferation and migration, but has no effect on cell

invasion in glioblastoma.[27] Other studies showed that DLG3 inhibited the growth and adhesion of cells by regulating β-catenin in an APC-independent manner.[19] DLG3 also appears to function in coupling NMDA receptors to the MAPK/ERK pathway.[28] Further research is needed to determine whether DLG3 functions in the progression of human malignant tumours *via* regulation of the MAPK/ERK pathway. DLG3 and several other polarity proteins are overexpressed in different cancers. For example, PAR6 is overexpressed in ER-positive breast cancer and non-small-cell lung cancer; aPKC and PKCζ are overexpressed in hepatocellular carcinoma, bladder tumours, and pancreatic cancer.[5] The results of these studies suggest that overexpression of polarity proteins may be a compensatory mechanism used to establish or maintain proper cell polarity.[29] Thus, the relationship of overexpressed DLG3 in cell polarity maintenance and malignant process of breast cancer needed to be studied in further research.

Previous research predicted that DLG3 is a secreted or plasma membrane protein, and the serum level has potential for use as a diagnostic tool.[21] However, further research is needed to assess the clinical applications of DLG3. Interestingly, up-regulation of miR-1246 reduces DLG3 expression,[30] so microRNA inhibition

TABLE 1 Relationship of DLG3 expression with clinical and pathologic parameters of breast cancer patients

	N	Expression level High	Expression level Low	χ^2	P
Tumour stage	155			27.09	<.0001
Stage 1	35	14	21		
Stage 2	74	19	55		
Stage 3	46	34	12		
Breast cancer subtype	155			8.44	3.70×10^{-3}
Luminal A/B & Her2+	98	51	47		
Triple-negative	57	16	41		

seems to be a promising approach to lower the expression of DLG3 in luminal A/B and Her2+ breast cancers.

In conclusion, DLG3 has higher expression in cancerous breast tissues than healthy breast tissues and higher expression in cancers with the luminal A/B and Her2+ subtypes than the triple-negative subtype. DLG3 expression also had a positive correlation with pathologic stage. We suggest that DLG3 might be useful as a diagnostic and prognostic indicator for breast cancer.

4 | MATERIALS AND METHODS

4.1 | Association of DLG3 mRNA with overall survival

UALCAN (http://ualcan.path.uab.edu/index.html) is a web tool used to analyze tumour transcriptome data. This web tool provides publicly accessible cancer transcriptome data (TCGA mRNA sequencing), with graphs and plots of gene expression, and information on patient survival. DLG3 mRNA expression in adjacent normal tissue and cancerous breast tissue specimens and Kaplan-Meier survival curves were compared using the UALCAN web tool.

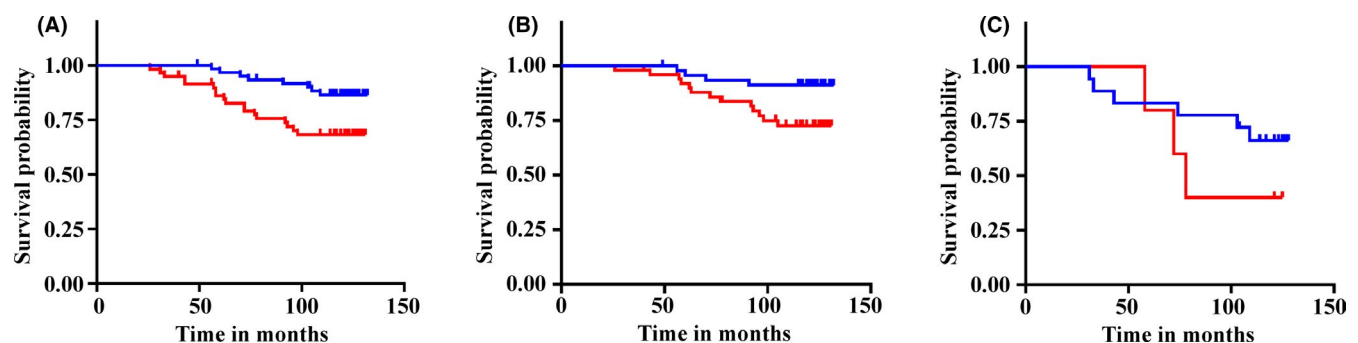## 4.2 | Association of DLG3 expression with pathological features

LinkedOmics (http://www.linkedomics.org/login.php) is a web tool used to analyze the multi-omics data from all 32 TCGA cancer types. LinkedOmics contains three analytical modules for identification and analysis of data on mRNA or protein expression signatures, biomarkers of clinical attributes, and putative target genes of transcriptional factors, microRNAs, and protein kinases. The association of DLG3 expression with pathological features in breast cancer was analyzed using LinkedOmics.[31]

4.3 | Tissue microarrays, patients, and follow-up

This study was approved by the Ethics Committee on Human Research of the First Affiliated Hospital of Xi'an Jiaotong University (Shaanxi, China). Tissue microarrays of 155 breast cancer tissues were obtained from Shanghai Biochip Co. (Shanghai, China). These samples were from patients with a mean age of 54 years (range: 29–87 years), and 121 of them were followed up for 26–131 months. The last follow-up data were collected from January 2005 to August 2016. The OS time was defined as the time from surgery to death or the last known follow-up. Ninety-five patients were still alive at the last follow-up.

4.4 | Immunohistochemistry (IHC) and scoring

Paraffin-embedded tissue microarray slides were deparaffinized in xylene, rehydrated in ethanol, washed with phosphate-buffered saline (PBS), and a 3% H_2O_2 solution was then used to block endogenous peroxidase activity. After antigen retrieval, goat serum was added to block non-specific binding sites, and the slides were incubated with the anti-DLG3 primary antibody (Proteintech, Wuhan, China) in a moist box at 4°C overnight. The slides were then rinsed, stained using the 3, 3′-diaminobenzidine (DAB) liquid chromogen substrate, counterstained with hematoxylin, and visualized using a Leica Microsystems slide scanner (SCN 400; Leica, Mannheim, Germany). Three randomly selected microscopic fields were

FIGURE 5 Association of DLG3 expression with OS among all breast cancer patients (A; $P = 1.20 \times 10^{-2}$, log-rank test), patients with luminal A/B or Her2 + subtypes (B; $P = 1.50 \times 10^{-2}$, log-rank test), and patients with triple-negative breast cancer (C; $P = .29$, log-rank test). A, Expression level: ● High expression (n = 59); ▲ Low expression (n = 62); B, Expression level: ● High expression (n = 51); ▲ Low expression (n = 47); C, Expression level: ● High expression (n = 5); ▲ Low expression (n = 18)

individually examined by blinded investigators in each IHC sample, and the intensity of staining was scored as 0 (negative), 1 (weakly positive), 2 (moderately positive), or 3 (strongly positive). The extent of IHC staining was then assessed according to the percentage of positive cells: 0 (<10%), 1 (10%–40%), 2 (40%–70%) and 3 (>70%). Then the IHC staining score of each field calculated as the product of the intensity and the extent of staining (range, 0–9). Expression was then scored as low (<5) or high (≥5).

4.5 | Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 7.00 (GraphPad, San Diego, CA, USA). The significance of a difference between two groups was determined using the Mann–Whitney test, and the significance of a difference among four groups was determined using the Kruskal–Wallis test. The significance of a difference in the survival rates of two groups was determined using the log-rank test. For the tissue microarrays, the significance of the association of DLG3 expression with pathological variables was determined using the Chi-square test. All statistical tests were two-sided and all results were expressed as the mean ± SEM for *$P < .05$, **$P < .01$, ***$P < .001$.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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