

Lactate Dehydrogenase-A-Forming LDH5 Promotes Breast Cancer Progression

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Background: Breast cancer (BC) has become the main malignant tumor threatening the health of women worldwide. Previous studies have reported that Lactate dehydrogenase-A (LDHA) has critical roles in cancer development and progression. We aimed to explore the roles of LDHA and LDH5 isoenzyme activity in BC, which provides a new insight into LDHA for the treatment of BC.

Methods: The expression of LDHA in BC and its relationship with clinicopathological features were obtained from various databases including The Cancer Genome Atlas (TCGA), Human Protein Atlas (HPA), Breast Cancer-Gene Expression Miner (bc-GenExMiner), TNMplot, UALCAN. The Kaplan–Meier Plotter was used to evaluate the prognostic value of LDHA. Western blot was performed to detect LDHA expression. Agarose gel electrophoresis was performed to detect the activities of LDH isoenzymes. The in vitro proliferation, migration and invasion potentials of BC cells were evaluated using MTT assays, colony formation, wound-healing assay, matrix metalloproteinase assays and transwell assays, respectively. The activities of LDH isoenzymes in serum and tissues were measured in patients with BC and healthy controls.

Results: Compared to normal tissues, LDHA expression was significantly higher in BC tissues. Patients' nodal status, histological types, *TP53* mutation status and PAM50 subtypes were significant factors influencing the *LDHA* expression. By overexpressing or silencing *LDHA* gene in BT549 cells, it was confirmed that LDHA promoted cell proliferation, migration and invasion. LDH5 isoenzyme activity in patients with BC was higher than healthy controls. The increased activity of LDH5 isoenzymes was induced by overexpression of LDHA in BC. High expression of LDHA was found to be associated with poor prognosis in BC.

Conclusion: LDHA plays a critical role in the progression of BC through the regulation of the activity of LDH5 isoenzyme, indicating that LDHA may serve as a valuable target for BC treatment.

Keywords: LDHA, LDH5 isoenzyme, breast cancer, progression, prognosis

Introduction

Breast cancer (BC) has become the main malignant tumor threatening the health of women worldwide.¹ Due to the limited number of identified targets, chemotherapy, surgery, and hormone treatments are commonly used, but they often entail significant side effects.² Therefore, it is urgent to develop novel molecular targets to help improve BC. Currently, increasing evidence has demonstrated that cancer is a metabolic disease.^{3,4} The “Warburg effect” indicates that under the condition of sufficient oxygen, tumor cells tend to rely on aerobic glycolysis for energy production. This seems to be an “uneconomic” way to obtain energy.⁵ Lactate dehydrogenase (LDH) is a crucial enzyme of glycolytic metabolic pathway. High LDH values is recognized as a major factor in the poor prognosis of BC.⁶

There are four subtypes of LDH: LDHA, LDHB, LDHC and LDHD. Among them, LDHA and LDHB are two significant isoforms of LDH, which are predominantly expressed in different types of cancer cells.⁷ LDHC is a typical cancer testicular antigen that can be used as a targetable cancer testis antigen for immunotherapy.⁸ LDHD promotes growth, migration, self-renewal, and tumorigenic potential in esophageal squamous cell carcinoma.⁹ However, many studies are still focused on LDHA, which favors the conversion of pyruvate to lactate.¹⁰ Acidic environment provides favorable conditions for tumor invasion.¹¹ A recent study revealed that LDHA promoted the proliferation, invasion, and migration abilities of renal cell carcinoma cell via upregulating the expression of EPHA2 in exosomes.¹² In addition, the up-regulation of LDHA in glioblastoma and pancreatic cancer has been confirmed to be correlated with poor prognosis of patients.^{13,14} Therefore, LDHA may be an important molecular target in BC.

LDH isoenzyme spectrum reflects the tissue-specific glucose metabolism status with the different contents of LDHA and LDHB subunits. There are five isoenzymes of LDH, corresponding to five possible combinations, named LDH1 (B4), LDH2 (B3A), LDH3 (B2A2), LDH4 (BA3), and LDH5 (A4) respectively.¹⁵ LDH5 is assembled only from subunit A. The activity of LDH5 isoenzyme in tumors is significantly increased and closely related to poor prognosis.¹⁶ Thus, it is interesting to investigate the role of LDHA and LDH5 isoenzyme in BC. It has been proposed that activity analysis of the LDH isoenzyme helps to reflect functions and energy requirements in different tissues.¹⁷ Changes in tissue or serum LDH isoenzymes are useful indicators of tissue damage in many diseases.¹⁸ Therefore, it is necessary to understand the composition and distribution of LDH isoenzymes in BC. However, the activities of LDH isoenzyme in BC remain unclear. In this study, we aim to explore the role of LDHA and LDH5 isoenzyme activity in BC, which provides a new insight into LDHA.

Materials and Methods

Gene Expression Analysis

The HPA database (<https://www.proteinatlas.org>) was used to explore the protein expression levels of LDHA in human pathology tissues. The RNA expression of *LDHA* in human mammary epithelial cell (HMLE) and various BC cell lines was also obtained through the HPA database. bc-GenExMiner (<https://bcgenex.ico.unicancer.fr/>) is a statistical mining tool. It was used to compare *LDHA* expression with clinical parameters such as nodal status, histological types, pathologic tumor stage and so on.^{19,20} The UALCAN database (<http://ualcan.path.uab.edu>) contains the gene expression data gathered from the TCGA database. We applied the website to analyze the differential expression of *LDHA* in BC tissues and normal tissues based on clinicopathological characteristics. The TNMplot online database (<https://tnmplot.com/analysis/>) was used to determine the expression of *LDHA* in pan-cancer tissues and normal tissues.²¹ The Kaplan–Meier plotter (<https://kmplot.com/analysis/>) was used to detect the correlation of LDHA protein expression and overall survival (OS) in BC patients.

Samples Collection

Human-tissue samples and serum samples were obtained from the Affiliated Hospital of Southwest Medical University from August 2021 to July 2022. Cancer and adjacent tissue samples were obtained from 12 patients with pathologically confirmed BC. A total of 69 serum samples of BC patients, as a case group, and 69 serum samples of healthy people, as a control group, the exclusion criterion were as follows: a history of malignancy in other organs, impairment of liver and kidney function. The study followed the 1964 helsinki Declaration and its later amendments or comparable ethical standards, all participants signed the informed consent.

Cell Lines, Culture and Transfection

The human BC cell lines MCF-7, BT-549, ZR-75-30, MDA-MB-231, SK-BR-3, T47D were obtained from the China center for type culture collection. MCF-7, MDA-MB-231, SK-BR-3, T47D were cultured in Dulbecco's modified Eagle Medium (DMEM, Gibco, USA), BT-549, ZR-75-30 were cultured in RPMI-1640 medium (Gibco, USA), supplemented with 10% fetal bovine serum (Gibco), respectively. All cell lines cultured at 37 °C in humidified air with 5% CO₂. Cells were transfected by Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions.

RNA Interference

LDHA and control siRNAs were all purchased from Gene Pharma (Shanghai, China). Two independent *LDHA* siRNAs gave similar results and efficiently silenced *LDHA*: *LDHA* siRNA 1(5'-GGAGUGGAAUGAAUGUUGC(dTdT)-3'); *LDHA* siRNA 2 (5'-CUGGCAAAGACUAUAAUGU(dTdT)-3').

Western Blot

Proteins from whole cells lysed in RIPA buffer, which were quantified by the BCA protein assay kit. Protein samples were separated by 10% SDS-PAGE electrophoresis and transferred onto PVDF membranes. Then membranes blocked with 5% nonfat milk in TBS Tween 20 at room temperature for 1 h and incubated with each primary antibody overnight at 4 °C. *LDHA* polyclonal antibody was purchased from Santa Cruz, used at a dilution of 1:300 (sc-137243); anti-beta-actin was purchased from Bioworld, used at a dilution of 1:10000 (AP0060). Next day, membranes were washed three times with TBST and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature (They were all purchased from Solarbio). Membranes were washed three times with TBST again. The immunoreactive protein bands were visualized by ECL detection system (ChemiScope 6000).

MTT for Cell Proliferation

Cells were seeded in a 96-well plate at a density of 5000 cells per well. After 4–6 h, cells were transfected and cultured for 48 h. Then, 20 μ L MTT (5 g/L) (purchased from Solarbio) was added to each well at 37 °C. After 4–6 h, the media was removed and 150 μ L DMSO was added to each well, absorbance values were obtained at 490 nm by using enzyme-labeled instrument (Bio Rad). Experiments were repeated three times and five parallel samples were measured each time.

Colony Formation

Transfected cells were seeded in 6-well plates at 1000 cells, 1500 cells, 2000 cells per well. The culture was replaced every three days. Three weeks later, the medium was removed, and 4% paraformaldehyde was added to each well for 20 min. Then the media was removed, cells were stained with 0.5% crystal violet for 30 min and we took pictures for record.

Wound Healing Assay

Cells were seeded in 24-well plates at a density of 2×10^5 /mL cells per well. After 24 h, cells were transfected and cultured to 80% density. Then the wound was created by 10 μ L pipette tip. Detached cells were washed by PBS, took pictures at once. After 24 h, 48 h, took pictures again.

Transwell

Transfected cells diluted into 1×10^6 /mL serum-free RPMI-1640 medium at a volume of 200 μ L for the transwell assay. Cells were seeded in the Matrigel-coated (BD Biosciences) upper chamber (Millicell), the lower chamber was loaded with RPMI-1640 medium containing 20% FBS at a volume of 600 μ L. After 24 h, the medium was removed, 4% paraformaldehyde was added to each chamber for 20 min. Then the media was removed, cells were stained with 0.5% crystal violet for 30 min and we took pictures for record.

Matrix Metalloproteinase Assays

Cells were cultured to 80% density and transfected. After 48 h, cells were washed three times with serum-free RPMI-1640 medium, and continue to culture for 24 h with serum-free RPMI-1640 medium. Then cell supernatant was collected and proteins were quantified by the BCA protein assay kit. Protein samples were separated by 0.1% SDS-PAGE electrophoresis. The gels were washed four times with eluent, each time for 15 min; washed twice with floating lotion, each time for 20 min; placed in the water bath for 48 h at 37 °C; stained for 3 h. The gels were placed in the decolorizing liquid A, B and C successively, washed for 0.5 h, 1 h and 2 h respectively. Lastly, bands were visualized by gel image analysis system (Bio Rad).

LDH Agarose Gel Electrophoresis

Transfected cells were washed twice with PBS and terminated with 1% TritonX-100. Then cell supernatant was collected and placed in the water bath for 30 min at 37 °C. The supernatant was centrifuged for 30 minutes at 16000 r/min, retrieved for supernatant again. Samples were separated by agarose gel electrophoresis, dyed in the water bath for 30 min at 37 °C, Lastly, bands were visualized by gel image analysis system (Bio Rad).

Statistical Analysis

The expression of *LDHA* in pan-cancer tissues and normal tissues was analyzed by Mann–Whitney. Wilcoxon test was utilized to discuss *LDHA* expression and its relationship with clinical features from the TCGA database. Student's *t*-test and ANOVA were used depending on experimental conditions. Survival analysis was performed using the Kaplan–Meier curve, and the differences were compared using the Log rank test. GraphPad Prism 7 and SPSS software (SPSS, version 19.0) was used for statistical analysis. All data presented as means ± SEM, $P < 0.05$ was considered statistically significant.

Results

LDHA Expression Was Higher in Breast Cancer Compared to Normal Tissues

To explore the potential roles of LDHA in tumors, we evaluated the gene expression of *LDHA* between cancer tissues and normal tissues by using TNMplot database. The results demonstrated that *LDHA* was highly expressed in most cancer tissues compared to the normal group (Figure 1A). Then we analyzed protein expression data of LDHA from the HPA databases. Our results confirmed LDHA exhibited strong or moderate expression levels across various types of malignancies including BC (Figure 1B). The results of UALCAN database analysis further confirmed that *LDHA* mRNA expression was higher in BC than that in normal tissues (Figure 1C). Collectively, these results suggested that LDHA was highly expressed in BC.

Relationships Between *LDHA* and Clinical Characteristics of BC Patients

We further analyzed the expression of *LDHA* in BC patients and its relationship to clinical characteristics from the TCGA database. Notably, *LDHA* expression was associated with patients' nodal status ($p = 0.0010$), histological types ($p < 0.0001$), *TP53* mutation status ($p < 0.0001$) and PAM50 subtypes ($p < 0.0001$) in BC, as illustrated in Table 1. These results were consistent with that obtained by UALCAN database. We found that *LDHA* was highly expressed in BC patients without node metastasis (Figure 2A). Furthermore, BC patients with histological types of invasive lobular carcinoma (ILC) showed lower expression of *LDHA* compared to others (Figure 2B). Triple negative carcinoma (TNBC) is a more aggressive type of BC,²² which had higher *LDHA* expression compared to Luminal (Figure 2C). Moreover, *LDHA* expression was higher in patients with *TP53* mutations compared to that with wild type status (Figure 2D). Existing studies have shown that *TP53* mutations are involved in the progression of various tumors.²³ This indicated higher expression of *LDHA* was associated with the function of *TP53* mutations in BC.

LDHA is Highly Expressed in Most Breast Cancer Cells

To analyze the significance of LDHA in BC cells, we analyzed RNA-seq data from non-tumorigenic HMLE and BC cell lines available in the HPA databases. The HPA database revealed that BC cell lines exhibited higher levels of *LDHA* expression compared to HMLE (Figure 3A). Then we detected the basic LDHA protein expression of six types of BC cells by Western blot. Results showed that LDHA was expressed in the six types of cell lines. Meanwhile, there was relatively higher LDHA expression in ZR-75-30, SK-BR-3, MDA-MB-231 and MCF-7 compared to BT549 and T47D (Figure 3B, the detailed data are presented in Supplementary Figure 1). In addition, we detected the activities of LDH isoenzymes in six BC cells. Interestingly, LDH5 isoenzyme was found in six types of BC cells and occupied different proportions in different types of cells. BT549 had lower activity of LDH5 isoenzyme compared to other cell lines but with all five LDHs isoenzymes present (Figure 3C). Therefore, BT549 was chosen for further study.

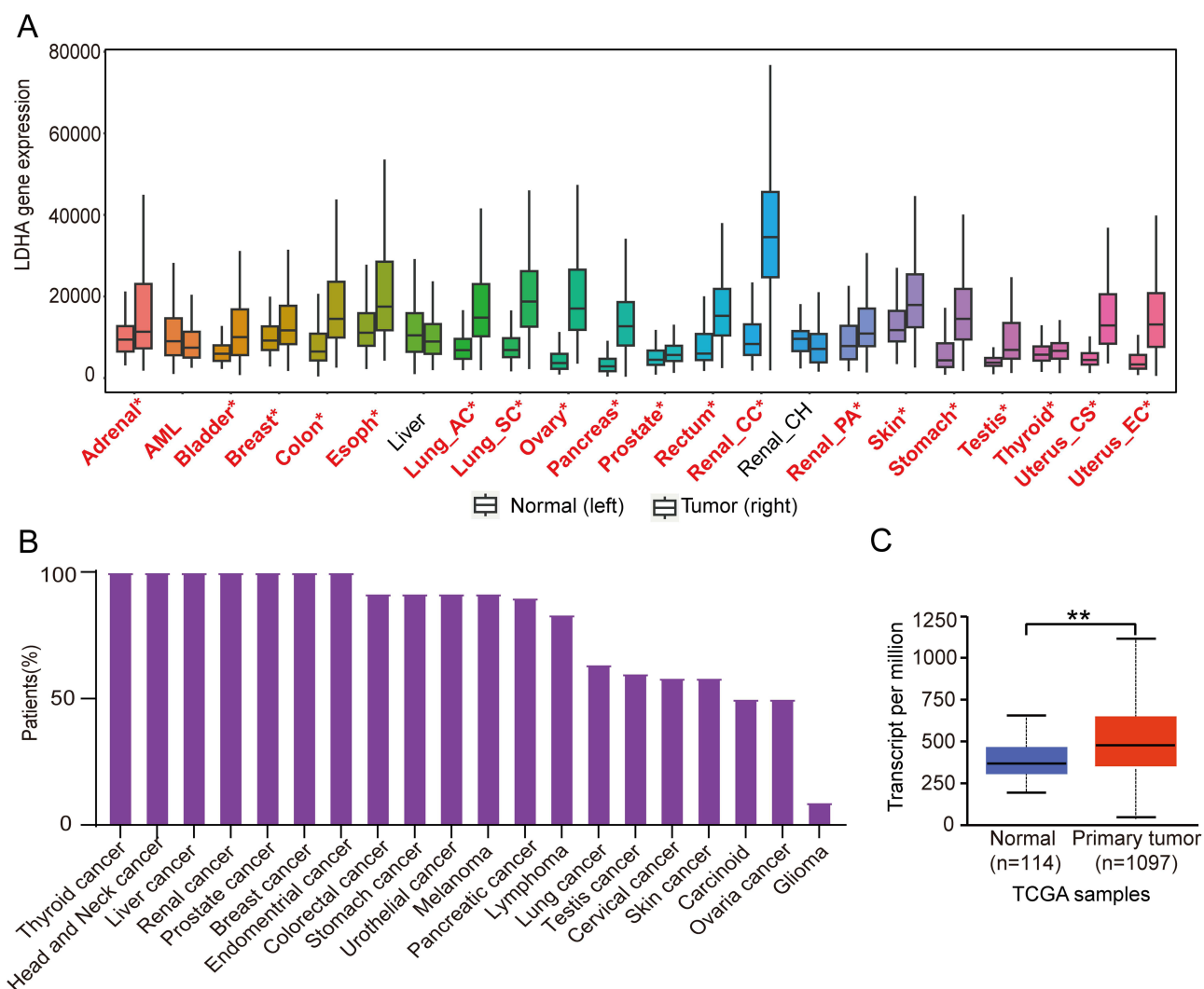


Figure 1 LDHA expression was higher in Breast Cancer compared to normal tissues. **(A)**, LDHA gene expression in pan-cancer tissues and normal tissues was analyzed by using TNMplot database. Significant differences are marked with red font; **(B)**, Protein expression of LDHA in various human pathology tissues was analyzed by using HPA databases (antibody: CAB015336). For each cancer, purple bars indicated the percentage of patients (maximum 12 patients) with high and medium protein expression level; **(C)**, LDHA expression in BC and normal samples from UALCAN database. * $p < 0.05$, ** $P < 0.01$.

LDHA Overexpression Promotes Breast Cancer Cell Proliferation, Migration and Invasion

To explore the potential oncogenic roles of LDHA, we transfected BT549 cells with the *LDHA*-expressing plasmid. The result showed a significant increase in LDHA expression compared to the control group (Figure 4A, the detailed data are presented in Supplementary Figure 2). As we expected, overexpression of LDHA significantly increased cell proliferation and colony formation (Figure 4B and C). Wound healing is a process based on the interactions between cell proliferation and migration. Overexpression of LDHA significantly increased the capacity of wound healing in BT549 cells (Figure 4D). Additionally, MMP2 and MMP9 are part of a family of proteolytic enzymes responsible for remodeling the extracellular matrix. MMP2 and MMP9 can promote cancer cell migration and invasion by degradation of type IV collagen of basement membranes.²⁴ It was found that overexpression of LDHA increased the activity of MMP-2 and MMP-9 indicating LDHA plays an important role in tumor migration and invasion (Figure 4E). Moreover, the number of migrating BT549 cells was significantly increased compared to the control group. (Figure 4F). Collectively, these findings demonstrated that the overexpression of LDHA promoted the progression of BC on cell proliferation, migration and invasion.

Table 1 Correlations Between the *LDHA* Expression and Clinical Characteristics of Patients with BC from the TCGA

Clinical Features	Case	LDHA Level (mean±SD)	p value
Nodal status			
N-	332	6.6845±0.6570	0.0010**
N+	358	6.2187±0.6568	
Histological types			
IDC	549	6.6845±0.6523	<0.0001**
ILC	116	6.2141±0.5015	
IDC&ILC	23	6.4055±0.5364	
Mucinous	13	6.7674±0.9949	
Pathologic tumor stage			
I	145	6.6545±0.5581	0.1987
II	440	6.6081±0.6823	
III	152	6.5218±0.7069	
IV	0	-	
Age			
[26,40]	74	6.6125±0.6809	0.7141
[40,70]	545	6.5923±0.6593	
[70,90]	124	6.6483±0.7003	
TP53 mutation status			
Wild type	477	6.4945±0.6035	<0.0001**
Mutated	252	6.8241±0.7359	
BRCA1/2			
Wild type	678	6.5902±0.6756	0.0956
BRCA1-mutated	28	6.7236±0.4657	
BRCA2-mutated	23	6.8417±0.6568	
PAM50 subtypes			
Basal-like	136	6.8106±0.7142	<0.0001**
HER2-enriched	51	6.9587±0.6178	
Luminal A	268	6.7275±0.5065	
Luminal B	281	7.1020±0.7192	

Notes: Bold means the *p*-value for that parameter is statistically significant. ** *P* < 0.01.

Abbreviations: BC, breast cancer; LDHA, Lactate dehydrogenase-A; TCGA, The Cancer Genome Atlas; HPA, Human Protein Atlas; bc-GenExMiner, Breast Cancer-Gene Expression Miner; LDH, Lactate dehydrogenase; HMLE, human mammary epithelial cells; IDC, Invasive Ductal Carcinoma; ILC, Invasive Lobular Carcinoma; Mucinous, Mucinous Carcinoma.

Silencing of LDHA Inhibits Breast Cancer Cell Proliferation, Migration and Invasion

Then, we continued to evaluate the capacity of proliferation, migration and invasion in BC cells transfected with siRNAs (*siLDHA-1* or *siLDHA-2*). The expression of LDHA in siRNAs groups was significantly reduced compared to the control group (Figure 5A, the detailed data are presented in Supplementary Figure 3). Silencing of LDHA significantly inhibited cell proliferation and colony formation (Figure 5B and C). In addition, silencing of LDHA significantly inhibited the capacity of wound healing in BT549 cells (Figure 5D). The activity of MMP-2 and MMP-9 were notably weakened by siRNAs transfection (Figure 5E). Moreover, transwell assay showed that the number of migrating BT549 cells was significantly reduced, and most of the cells were smaller compared to the control group (Figure 5F). Therefore, these data indicated that silencing of LDHA inhibited cell proliferation, migration and invasion.

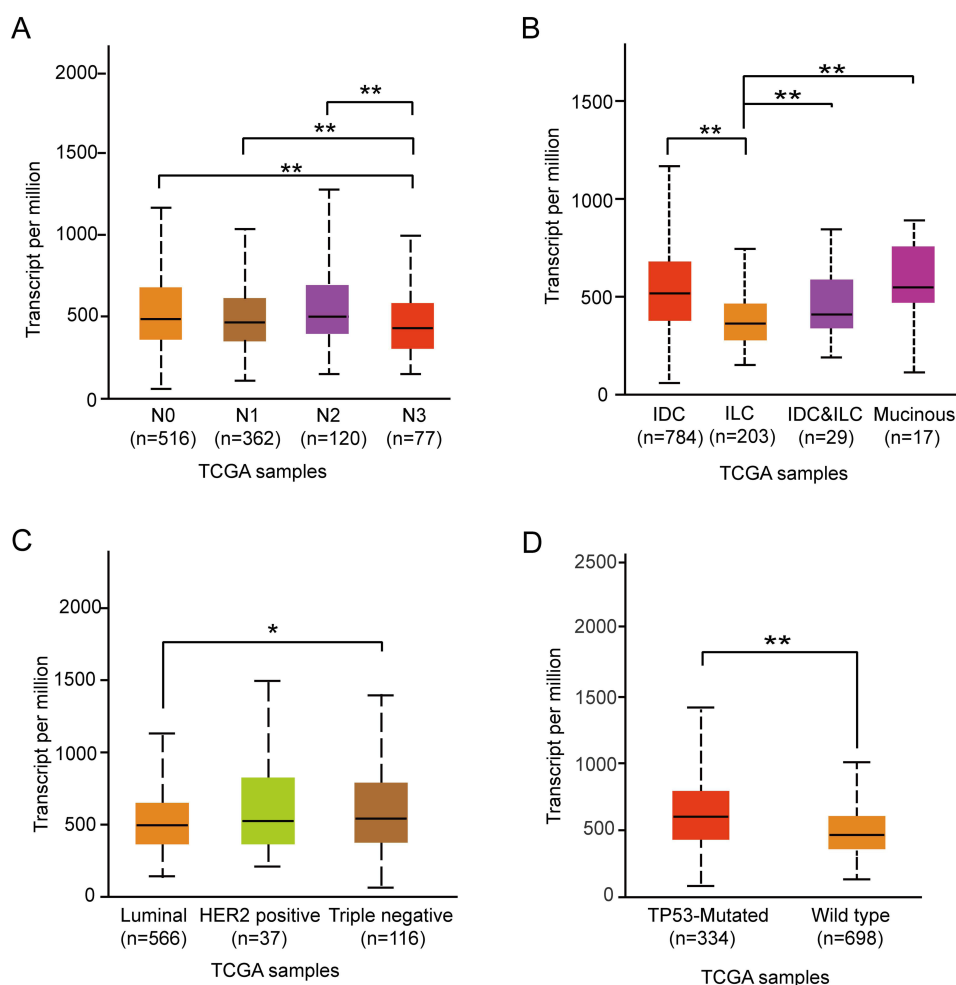


Figure 2 Relationships between *LDHA* and clinical characteristics of BC patients. The expression of *LDHA* in BC patients and its relationship to clinical characteristics from the UALCAN database. **(A)**, Expression of *LDHA* in BC based on nodal metastasis status; **(B)**, Expression of *LDHA* in BC based on histological types; **(C)**, Expression of *LDHA* in BC based on subclasses; **(D)**, Expression of *LDHA* in BC based on *TP53* mutation status. * $P < 0.05$, ** $P < 0.01$.

LDHA Expression is Associated with the Activity of LDH5 Isoenzyme

To further analyze the relationship between *LDHA* expression and LDH5 isoenzyme activity, we detected the activities of LDH isoenzymes. As we expected, overexpression or knockdown of *LDHA* expression mainly affected LDH5 isoenzymes. The LDH5 isoenzyme activity was prominently increased after *LDHA* overexpression (Figure 6A, the detailed data are presented in Supplementary Figure 4), while decreased after knockdown of *LDHA* (Figure 6B, the detailed data are presented in Supplementary Figure 5). LDH1 isoenzyme consisted of LDHB subunits. LDH2 isoenzyme contained three LDHB subunits and one LDHA subunit. Importantly, LDH1 and LDH2 were least affected when overexpression or knockdown of *LDHA*. However, the activities of LDH1 and LDH2 isoenzymes were increased after the knockdown of *LDHA*. We speculated that the relative increase in activities of LDH1 and LDH2 isoenzymes was due to the decrease of LDH5 isoenzyme. There were little changes in the activities of LDH3 and LDH4 isoenzymes. Therefore, these results indicated that *LDHA* expression determined the activity of the LDH5 isoenzyme in BC.

LDH5 Isoenzyme Activity is Higher in BC Patients

Once tissue was damaged, LDH is released into the circulation, contributing toward abnormal changes in the activities of LDH isoenzymes.²⁵ However, the tissues and serum activities of LDH isoenzymes in BC remain unclear. To investigate the activities of LDH isoenzymes in BC patients, we used agarose gel electrophoresis assay

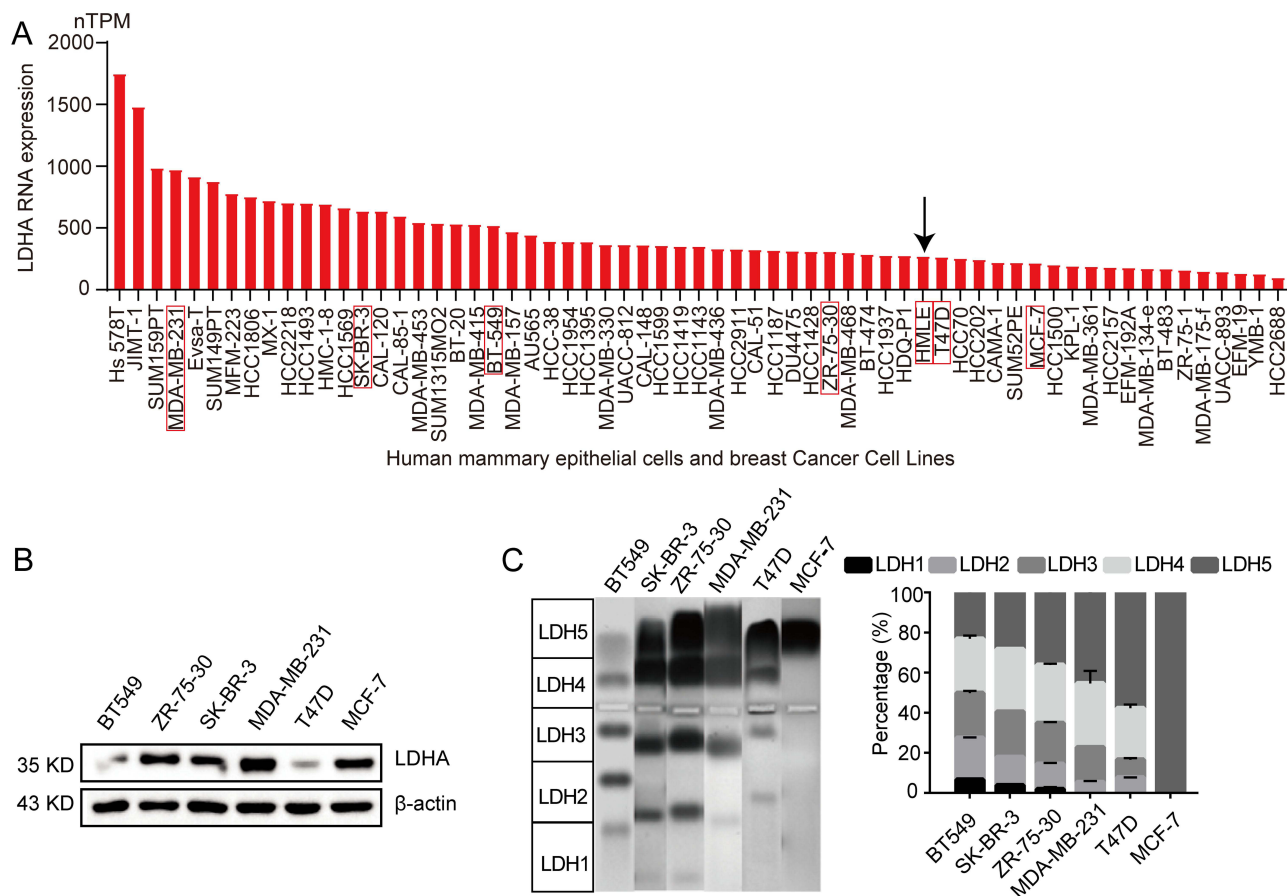


Figure 3 LDHA is highly expressed in most breast cancer cells. (A), RNA-seq data of LDHA in non-tumorigenic HMLE and various BC cell lines was analyzed by using HPA databases. The red box with the black arrow represented HMLE cells; (B), Western blot analysis of the expression of LDHA in six different BC cell lines; (C), Agarose gel electrophoresis assays of six different BC cell lines (n=3). Data are presented as the mean ± s.e.m.

to detect 12 pairs of BC tissues and adjacent normal breast tissues (Supplementary Figure 7A-J). The results showed that LDH isoenzyme in adjacent tissues consists of LDH1 to LDH4, and LDH5 isoenzyme was almost invisible. In contrast, LDH5 isoenzyme activity was detected in BC tissues and occupied a certain proportion (Figure 7A). Specifically, the activity of LDH5 isoenzyme in BC tissues increased to 18.84±8.07% compared to adjacent tissues (Figure 7B).

We further detected the activities of LDH isoenzymes in serum samples from 138 subjects (69 BC patients and 69 healthy controls) (Supplementary Figure 7A-P). The results demonstrated that serum LDH5 isoenzyme activity (13.41±3.05%) was higher in BC patients compared to healthy controls (12.07±2.13%), and the activity of the other isoenzymes had different degrees of decrease or increase (Figure 7C and D). Moreover, analysis of the statistical results showed that the increase of LDH5 isoenzyme activity was statistically significant (P<0.01) (Figure 7E). The experimental results of serum were similar to those of tissues. The above results indicated that LDHA played critical roles in BC, and the increased activity of LDH5 isoenzymes was induced by over-expression of LDHA in BC.

Compared to healthy controls, the serum LDH levels in BC patients were significantly increased (Figure 7E). However, the mean of serum LDH levels in BC patients was considered to be within the normal detection range (100–300 U/L) in clinical diagnosis, which suggested that LDH5 isoenzyme activity was a better biomarker than serum LDH levels for the diagnosis of BC. We also analyzed the Kaplan–Meier survival curves, BC patients with higher LDHA expression showed poor OS [hazard ratio (HR) = 2.14, p = 0.018]. In addition, in patients

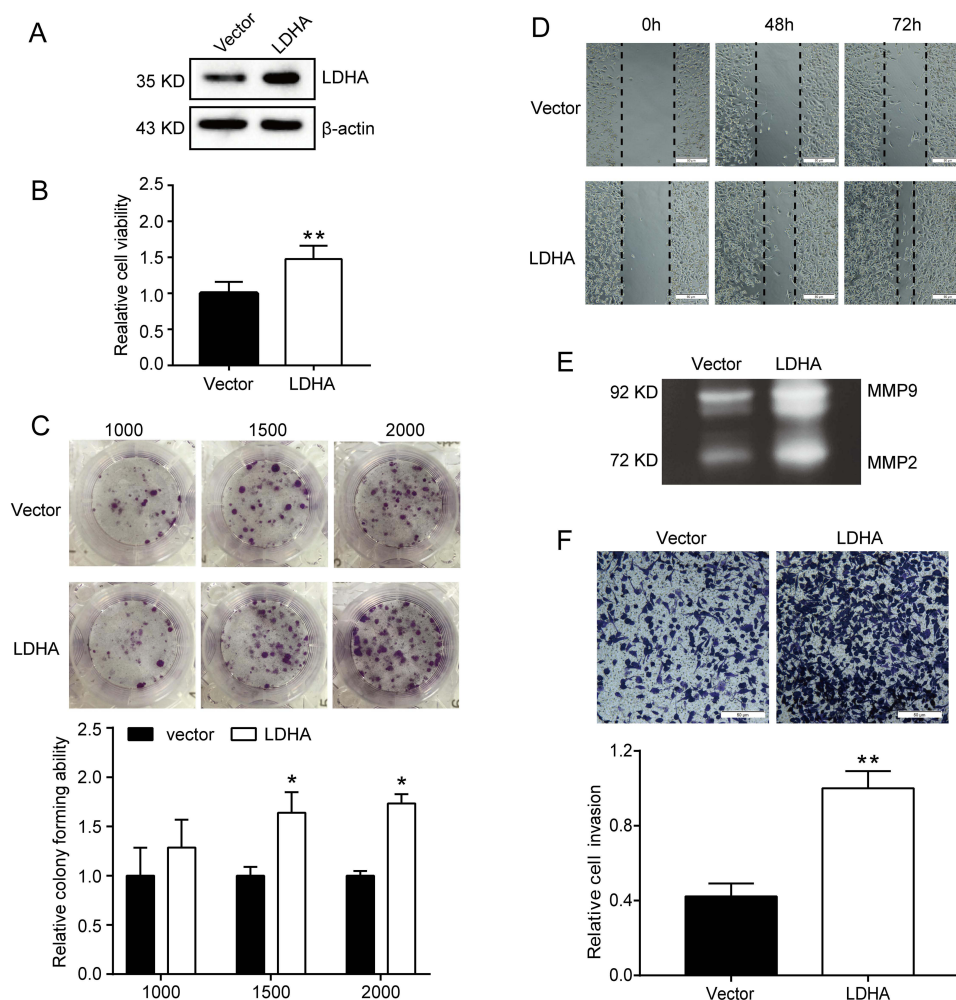


Figure 4 LDHA overexpression promotes breast cancer cell proliferation, migration and invasion. **(A)**, Western blot analysis of the expression of LDHA in BT549 cells transfected with LDHA-expressing plasmid; **(B)**, Cell proliferation was determined by MTT assay after transfection 48 h (n=5); **(C)**, Colony formation assay was performed to determine the colony forming ability in BT549 cells transfected with LDHA-expressing plasmid (n=3); **(D)**, Cell proliferation and migration were assessed by wound healing assay; **(E)**, The activity of MMP-2 and MMP-9 was assessed by gelatin zymography analysis; **(F)**, Cell invasion capacity was assessed by transwell assay (n=3). Data are presented as the mean \pm s.e.m. * $P < 0.05$, ** $P < 0.01$.

with positive lymph node status, high LDHA expression had a significantly negative effect on OS (HR = 3.38, $p = 0.021$) (Figure 7F). This indicated that LDHA was a risk factor for prognosis in patients with BC.

Discussion

Aerobic glycolysis is considered a key metabolic process involved in tumor growth.²⁶ The molecular expression changes are closely related to metabolic reprogramming, which can be used to track the occurrence and development of tumors. Along this line, Much literature has revealed that LDHA is critically involved in tumor development.^{27,28} Our study showed that LDHA expression was significantly higher in BC tissues compared to normal tissues. The tumor subclass types of TNBC and patients with *TP53* mutations tended to have higher expression of LDHA. TNBC is characterized by a high level of cell invasiveness and distant metastasis rates,²⁹ which may indicate that high expression of LDHA is a poor prognostic factor in BC. Then we investigated the oncogenic role of LDHA in BC, the results showed that LDHA was highly expressed in most BC cells. Various small-molecule LDHA inhibitors and siRNA-mediated knockdown of LDHA have been reported to inhibit its LDH enzymatic activity, thereby suppressing glycolysis and tumor progression.^{30–33} Moreover, Juan Liu and colleagues reported that targeting LDHA activity and Rac1 activity simultaneously displays a promising therapeutic strategy for BC with LDHA

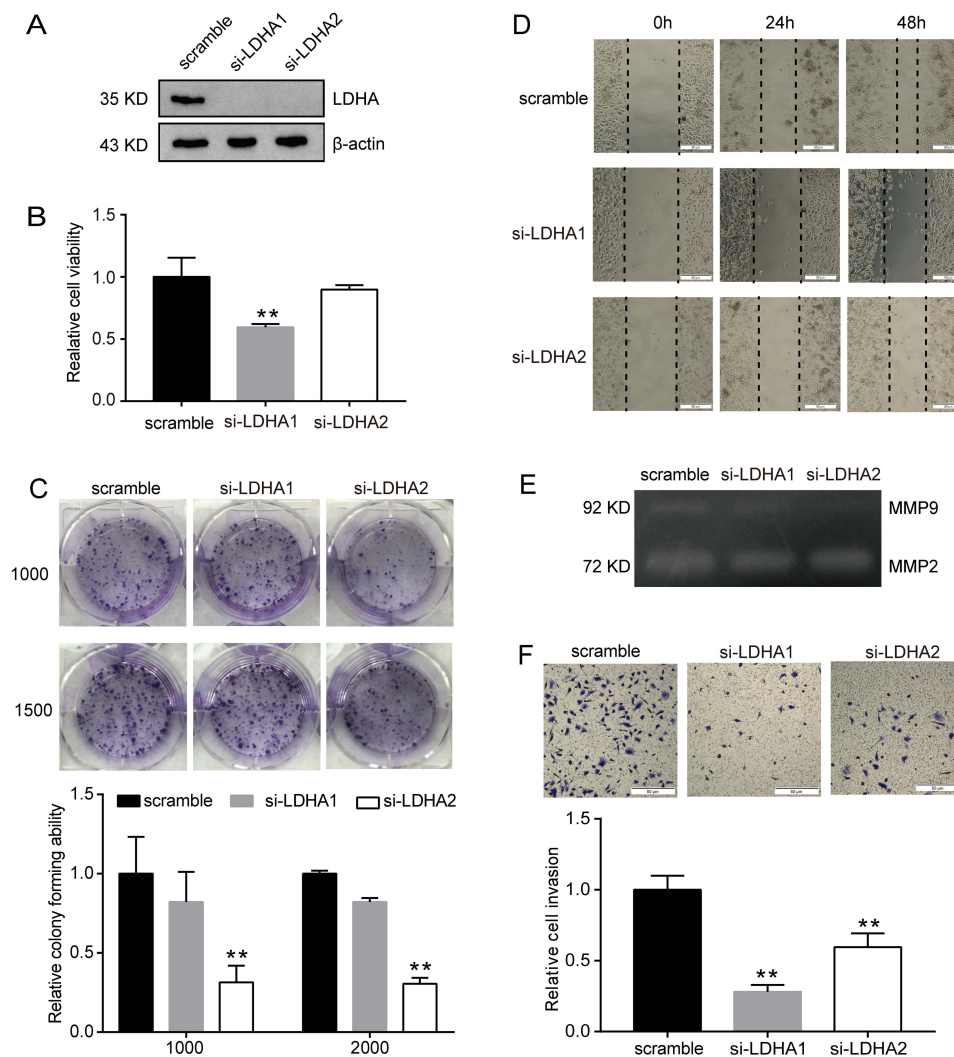


Figure 5 Silencing of LDHA inhibits breast cancer cell proliferation, migration and invasion. (A), Western blot analysis of the expression of LDHA in BT549 cells transfected with siRNA; (B), Cell proliferation was determined by MTT assay after siRNA transfection 48 h (n=5); (C), Colony formation assay was performed to determine the colony forming ability in BT549 cells transfected with siRNA (n=3); (D), Cell proliferation and migration were assessed by wound healing assay; (E), The activity of MMP-2 and MMP-9 was assessed by gelatin zymography analysis; (F), Cell invasion capacity was assessed by transwell assay (n=3). Data are presented as the mean \pm s.e.m. ** $P < 0.01$.

overexpression.³⁴ In our study, in vitro experiments demonstrated that overexpression of LDHA promoted cell proliferation, migration and invasion, while downregulation of LDHA suppressed proliferation migration and invasion in BC cells, indicated that LDHA has a tumor-promoting effect. Studies have demonstrated that LDH5 isoenzyme, encoded by *LDHA*, was considered as a vital therapeutic target for highly glycolytic human cancers. It was observed that overexpression of the *LDH5* gene conferred the growth advantages of highly glycolytic cancer cells, which correlated with the aggressive phenotypes and poor prognosis in several tumors.³⁵ However, few articles have reported on the role of LDH5 in BC. Our study revealed that the change in LDH5 isoenzyme activity was due to the expression of LDHA. Therefore, LDHA promotes the growth of BC cells by up-regulating the activity of LDH5 isoenzyme.

Moreover, the major type of LDH isoenzyme expressed in a particular tissue depends on its metabolic demands.³⁶ It was the first time that the LDH5 isoenzyme activity was detected in BC tissues, and occupied nearly 19% proportion compare to adjacent tissues. Serum LDH5 isoenzyme activity was higher in BC patients compared to healthy controls. These findings intuitively demonstrated that the LDHA expression was significantly up-regulated in BC, and the increased activity of LDH5 isoenzymes was induced by overexpression of LDHA. Furthermore, the activities of LDH isoenzymes could help to identify the main source of tissue damage. LDH1 was predominantly expressed in myocardial

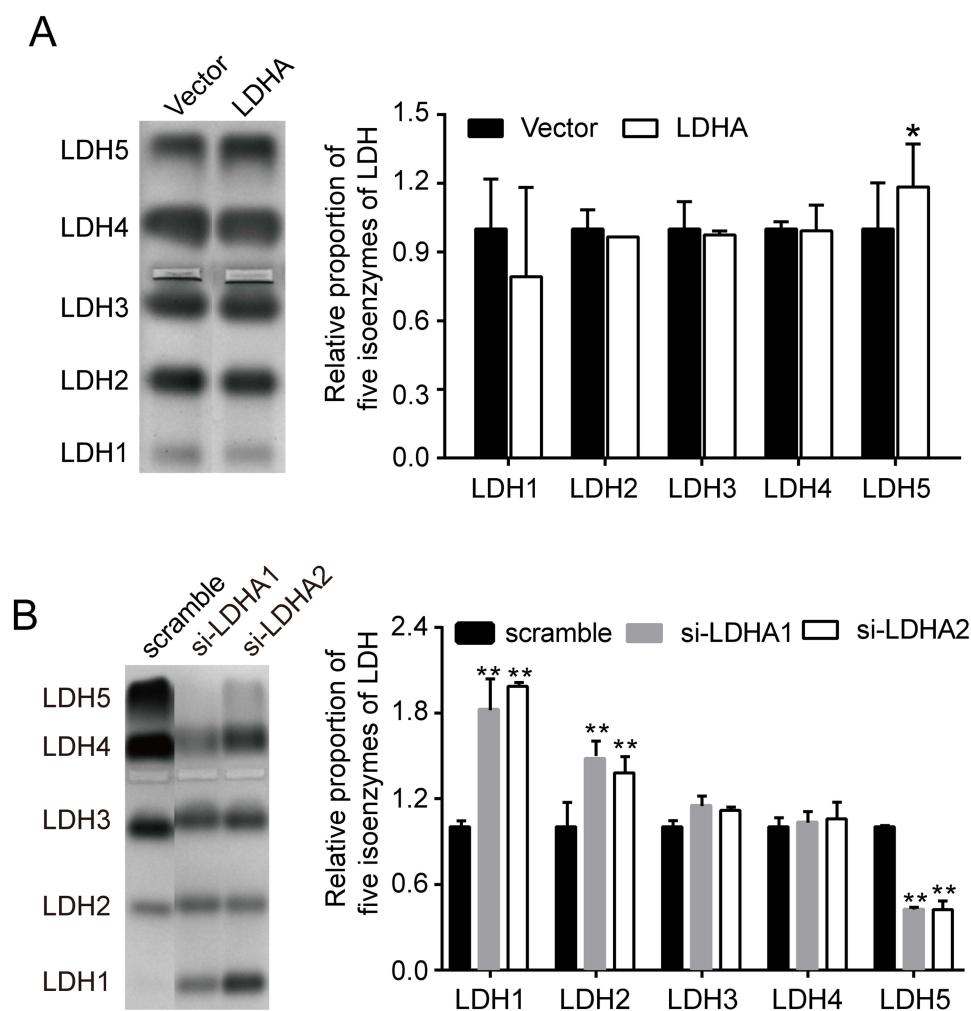


Figure 6 LDHA expression is associated with the activity of LDH5 isoenzyme. **(A)**, Agarose gel electrophoresis assay of BT549 cells transfected with LDHA-expressing plasmid (n=3); **(B)**, Agarose gel electrophoresis assay of BT549 cells transfected with siRNAs (siLDHA-1 or siLDHA-2) (n=3). Data are presented as the mean \pm s.e.m. * $P < 0.05$, ** $P < 0.01$.

damage, and LDH3 was predominantly expressed in pulmonary inflammation in both vascular and infectious pathologies.³⁷ We found the change in the activity of LDH5 isoenzyme was most prominent, suggesting that LDH5 is useful for the diagnosis of BC.

LDH in the serum is widely used for the assessment of risk stratification and prognosis in many cancers compared to LDH5 isoenzyme activity.^{38–40} However, some studies have revealed serum LDH levels do not always reliably reflect serum LDH5 isoenzyme activity.⁴¹ In our study, we found some patients have been diagnosed with BC, but without abnormal LDH in the serum. However, serum LDH5 isoenzyme activity was significantly higher compared to healthy controls. These findings highlighted the importance of LDH5 isoenzyme in the BC diagnosis. In addition, Kaplan–Meier analysis showed BC patients with high LDHA expression had poorer prognosis, which suggested that LDHA played an important role in the prognosis of BC.

Conclusions

In summary, we identified a new horizon to understand the role of LDHA in BC, suggesting the possibility that LDHA most likely promoted BT549 cell proliferation, migration and invasion by directly regulating LDH5 isoenzyme activity. The studies highlighted the importance of LDHA as an ideal target in BC, and a combination of LDHA expression and LDH5 isoenzyme activity can be used for early diagnosis and prognosis of BC.

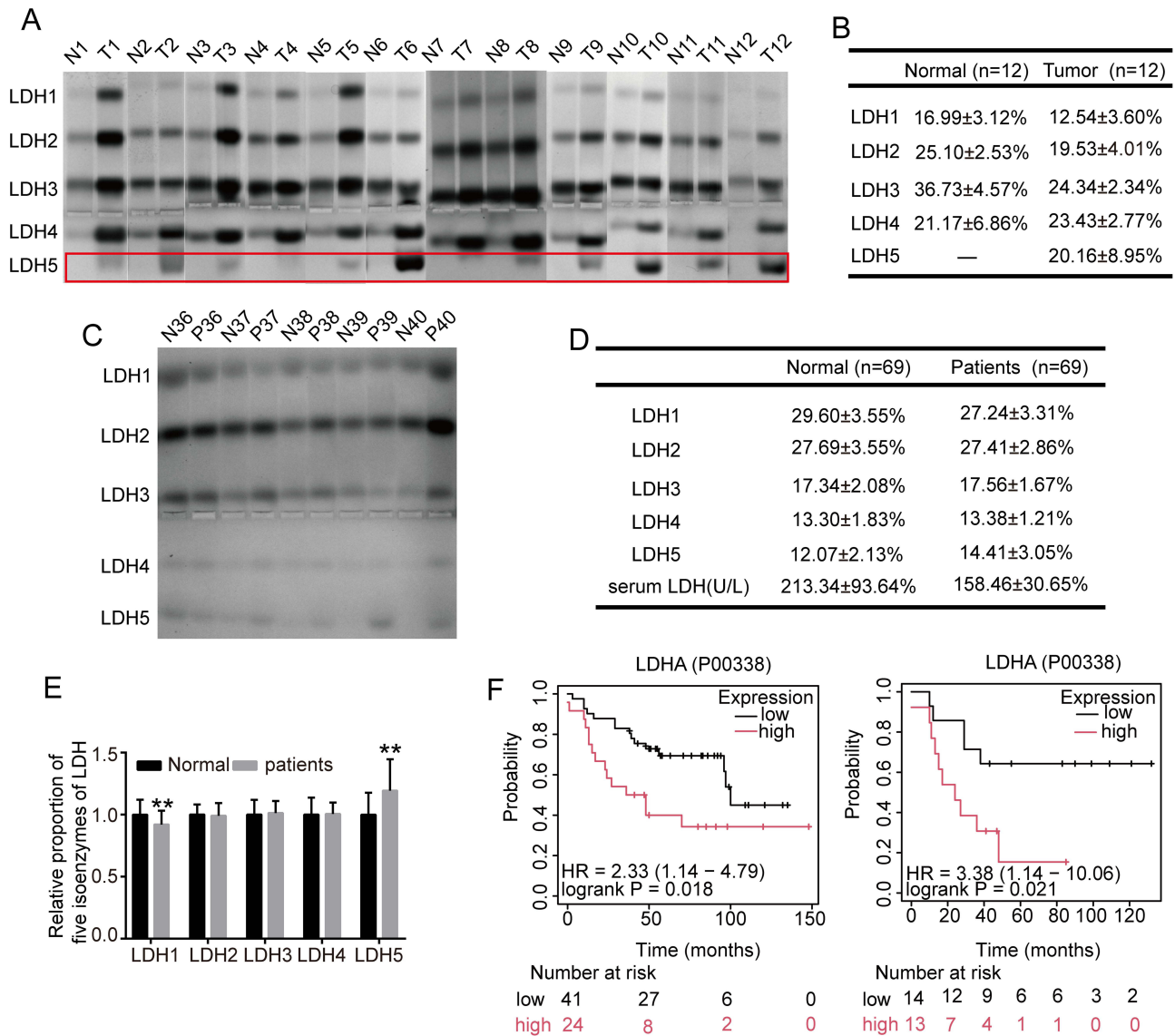


Figure 7 LDH5 isoenzyme activity is higher in BC patients. (A), The activities of LDH isoenzymes in 12 pairs BC tissues and their adjacent normal breast tissues; (B), Comparison of LDH isoenzyme activity between BC tissues and adjacent normal breast tissues; (C), Representative Diagram of agarose gel electrophoresis assay of serum samples, all results (69 BC patients and 69 healthy controls) obtained from [Supplementary Figure 7A-P](#); (D), Comparison of LDH isoenzyme activity and serum LDH levels between BC Patients and healthy controls; (E), The relative activity of LDH isoenzymes and serum LDH levels between BC Patients and healthy controls; (F), Kaplan–Meier Plotter database analysis of OS curves of patients with BC in relation to the LDHA levels with all subtypes (left) and with positive lymph node status (right). Data are presented as the mean ± s.e.m. ** $P < 0.01$.

Data Sharing Statement

The data that support the findings of this study are available on request from the corresponding author (Fancai Zeng, zfcgai@swmu.edu.cn).

Ethics Statement

The human samples used in this study conformed to national and institutional ethical guidelines and were approved by the Clinical Research Ethics Committee of Affiliated Hospital of Southwest Medical University (No. KY2021205). The studies were conducted in accordance with the local legislation and institutional requirements. All participants signed the informed consent.

Consent for Publication

All authors have consented to the publication of the manuscript.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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