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Coexpression of EphA10 and Gli3 promotes breast cancer cell proliferation, invasion and migration

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ABSTRACT

This study investigated the influences of EphA10 and Gli3 on breast cancer (BC) cell proliferation, invasion and migration. Immunohistochemistry was used to reveal the expressions of EphA10 and Gli3 in 18 intraductal carcinomas, 124 invasive carcinomas, 50 paracancerous tissues (2 cm away from the tumor, when possible or available), 50 lobular hyperplastic tissues and 30 normal breast tissues. qRT-PCR and Western blotting were applied to detect the expressions of EphA10 and Gli3 in invasive BC cells (MDA-MB-231, BT20 and Hs578T) and normal human mammary epithelial cells (MCF10A). MDA-MB-231 and BT20 cells were transfected with sh-EphA10, sh-Gli3 or sh-EphA10+sh-Gli3. CCK-8 was used to test the proliferation of transfected MDA-MB-231 and BT20 cells. Transwell and scratch assays were used for evaluation of invasion and migration of the transfected cells. EphA10 and Gli3 were highly expressed in invasive carcinomas and invasive BC cells. The expressions of EphA10 and Gli3 were associated with the clinicopathological characteristics and poor prognosis of patients with invasive BC. Knockdown of EphA10 or Gli3 suppressed activities of BC cells. Knockdown of both EphA10 and Gli3 was more effective than knockdown of Gli3 alone. Taken together, coexpression of EphA10 and Gli3 promotes BC cell proliferation, invasion and migration.

Significance of this study

What is already known about this subject?
- Breast cancer is a highly invasive and heterogeneous malignancy that results in vast mortality.
- EphA10 is highly expressed in breast cancer.
- Gli proteins are implicated in various tumorigenic processes.

What are the new findings?
- EphA10 and Gli3 were both highly expressed in invasive breast carcinomas and cells.
- Positive expressions of EphA10 and Gli3 are associated with poor prognosis of invasive breast cancer.
- EphA10 and Gli3 work in synergy to promote proliferation, migration and invasion of breast cancer cells.

How might these results change the focus of research or clinical practice?
- EphA10 and Gli3 can be concurrently targeted for treatment of breast cancer.

INTRODUCTION

In United States, breast cancer (BC) accounts for about 29% of all new cancer cases in women annually.[1] Tumor invasion and metastasis are dynamic multistep processes, which contribute to the vast majority of BC-associated mortalities.[2 3] Invasive ductal carcinoma is the most prevalent type of BC, followed by invasive lobular carcinoma and other rare types. Both invasive ductal and lobular BC can be diagnosed by pathological findings. However, the underlying causes of the occurrence and development of BC remain a mystery.[4] BC is heterogeneous in molecular changes, clinicopathologic characteristics, therapeutic responses and clinical outcomes. The initiation of BC is a complicated process characterized by genetic and epigenetic alterations that activate cellular pathways for tumorigenesis.[5] In spite of notable advances in early detection and diagnosis of BC, substantial challenges still remain in preventing the cancer initiation and improving the treatment of BC.[6] In China, the mortality of patients with BC has doubled over the last three decades.[7] Further exploration of the progression of BC is urgently needed to improve patients' survival.[8]

Eph receptor tyrosine kinases together with their ligands known as ephrins are involved in a large spectrum of developmental processes.[9] The Eph/ephrin signaling mediates cell-cell interaction and contributes to the tumorigenesis, angiogenesis and metastasis in many cancers.[10] Eph receptor A10 (EphA10) was characterized as a more efficient biomarker than the established prognostic marker Her-2 in BC.[11] EphA10 was highly expressed in various BC subtypes, and an anti-EphA10 antibody could significantly suppress tumor growth.[12] Moreover, both mRNA and protein expressions of EphA10 were positively correlated with tumor metastasis in BC.[13] Nevertheless, the function of EphA10 in BC cells is uncharacterized.

The Hedgehog (Hh) signaling pathway, which is well conserved in mammals and other vertebrate species, has long been known to regulate growth and patterning during embryonic development. Hh signaling pathways consist of Hh proteins (Sonic Hh, Indian Hh, and Desert Hh), 12 transmembrane protein patched (patched 1 and patched 2), 7 transmembrane protein smoothened (Smo) and 5-zinc finger transcription factor Gli proteins (Gli1, Gli2 and Gli3).[14 15] The Gli proteins are large and multi-functional transcription factors. The three members of Gli proteins behave differently with partially redundant functions and are implicated in tumorigenesis. Available data has supported the positive correlation of Gli3 with the progression of hepatocellular carcinoma,[16] colon cancer,[17] non-small-cell lung cancer,[18] prostate cancer[19] and cervical cancer.[20] However, the role of Gli3 in BC remains to be clarified.

The expressions of EphA10 and Gli3 were investigated in surgically resected BC tissues and cultured BC cells in this study. Synergic effect of EphA10 and Gli3 was observed in the malignant progression of BC.

MATERIALS AND METHODS

Patients and tissue samples

One hundred and twenty-four BC tissues, 50 pericancerous tissues, 50 lobular hyperplastic tissues and 30 normal breast tissues from benign lesions were obtained at the Second and Third Xiangya Hospitals, Central South University, from January 2000 to December 2002 and were histologically identified by two pathologists. The tumors were restaged according to the seventh TNM Classification of Malignant Tumors. Classification and histological grade of the cancerous tissues were determined with reference to the WHO classification of tumors.

Clinicopathological data of the intraductal carcinomas and invasive carcinomas are summarized in table 1. Survival data of the 124 patients with invasive BC were collected. The follow-up time for patients was 13 years. Twenty-six

Table 1 Association between clinicopathological characteristics (CPC) and tumor types

CPC	Case number	Intraductal carcinoma Positive number (%)	Invasive carcinoma Positive number (%)	χ^2	P value
Age (years)					
≤45	81	13 (72.7)	68 (54.8)	1.938	0.164
>45	61	5 (27.8)	56 (45.2)		
Menopausal status					
Premenopausal	80	9 (50.0)	71 (57.3)	0.337	0.562
Postmenopausal	62	9 (50.0)	53 (42.7)		
Pathologic types					
I	22	5 (27.8)	17 (13.7)	11.132	0.004
II	61	12 (66.7)	49 (39.5)		
III	59	1 (5.6)	58 (46.8)		
Tumor size					
≤3 cm	72	15 (83.3)	57 (46.0)	8.87	0.003
>3 cm	70	3 (16.7)	67 (54.0)		
ER					
+	73	14 (77.8)	59 (47.6)	5.738	0.017
–	69	4 (22.2)	65 (52.4)		
PR					
+	79	15 (83.38)	64 (51.6)	6.408	0.011
–	63	3 (16.7)	60 (48.4)		
CerB2					
+	85	7 (38.9)	78 (62.9)	3.773	0.052
–	57	11 (61.1)	46 (37.1)		
Lymph node metastasis					
No	73	17 (94.4)	56 (45.2)	15.283	0.000
Yes	69	1 (5.6)	68 (54.8)		
TNM stage					
I+II	83	17 (36.4)	66 (36.4)	10.081	0.014
III+IV	59	1 (1.7)	58 (70.3)		
EphA10					
+		8 (44.4)	76 (61.3)	1.846	0.174
–		10 (55.6)	48 (38.7)		
Gli3					
+		9 (50.0)	69 (55.6)	0.202	0.653
–		9 (50.0)	55 (44.4)		

ER, estrogen receptor; PR, progesterone receptor; TNM, tumor lymph node metastasis.

patients who survived longer than 13 years were included in the analysis as censored cases.

Pericancerous tissues were collected from the above 50 patients with invasive BC. The age of these 50 patients ranged from 32 to 70 (46.5±9.4) years. The pathological examination showed 18 normal breast tissues, 14 mild dysplasia, 10 moderate dysplasia and 8 severe dysplasia cases. Lobular hyperplastic tissues were collected from 50 patients aged from 28 to 60 (36.7±8.4) years. The pathological examination showed 16 normal lobular tissues, 19 mild dysplasia, 10 moderate dysplasia and 5 severe dysplasia cases. Normal breast tissues were collected from normal tissues beside 30 breast fibroadenoma and pathologically identified.

All tissues were made into paraffin sections for immunohistochemistry.

Immunohistochemistry

EnVision Detection Kit (Dako, Carpinteria, California, USA) was used in the experiment. Briefly, the paraffin-embedded tissues were cut into 4 μM thick sections. Deparaffinized tissue sections were treated with 3% H_2O_2 in dark for 15 min, followed by heat-induced epitope retrieval using sodium citrate buffer at 96°C for 30 min. Primary rabbit anti-human EphA10 and Gli3 antibodies (1:100, Dako) were incubated with the tissues for 2 hours. The tissues were treated with Solution A for 30 min before 3,3′-diaminobenzidine staining and hematoxylin counterstaining. Ten random fields of each section (500 cells per field) were examined by two observers independently. The final data took an average of the two observations. Positive cases were represented by 25% or more positive cells.

Cell culture

Invasive BC cell lines (MDA-MB-231, BT20 and Hs578T) and normal human mammary epithelial cells (MCF10A) were provided by Shanghai Institute of Cell Research, Chinese Academy of Sciences. Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, New York, USA) used for cultivation of the cells were supplemented with 10% fetal bovine serum (FBS), 1% penicillin and 1% streptomycin.

Cell transfection

MDA-MB-231 and BT20 cells were transfected with sh-EphA10 (20 µL), sh-Gli3 (20 µL) or the negative control (sh-NC, 20 µL) (GenePharma, Shanghai, China) using LipoFiter reagent (Hanbio, Shanghai, China). The following experiments were conducted 24 hours after triplicate transfection.

qRT-PCR

TRIzol reagent (Invitrogen, Carlsbad, California, USA) was used for extraction of total RNA from cells. Concentration and purity of the RNA samples were measured. The concentration of qualified RNA samples was adjusted, and the RNA was reverse-transcribed using a reverse transcription kit (Takara, Tokyo, Japan) and random primers according to the instructions. Gene expression was detected by LightCycler 480 (Roche Diagnostics, Indianapolis, Indiana, USA), and the reaction conditions were set according to the instruction of the fluorescent quantitative PCR kit (SYBR Green Mix, Roche Diagnostics). Each sample had three duplicates. The internal reference gene of mRNA was GAPDH. The $2^{-\Delta\Delta Ct}$ method was used for data analysis. Sequences of the primers are listed in table 2.

Table 2 Primer sequences

Name of primers	Sequences
GAPDH-F	GCAAGGATGCTGGCGTAATG
GAPDH-R	TACGCGTAGGGGTTTGACAC
EphA10-F	CCTGGTTAGGGCAGCGTTTA
EphA10-R	CTGACTGGAGTGGCTGAGTC
Gli3-F	GGCCATCCACATGGAATATC
Gli3-R	TGAAGAGCTGCTACGGGAAT

F, forward; R, reverse.

Western blotting

Cells lysed in radioimmunoprecipitation assay lysis buffer (Beyotime, Shanghai, China) were centrifuged for protein extraction. The protein concentration of each sample was measured using a BCA kit (Beyotime) to ensure the same loading amount of each protein sample. Corresponding volume of proteins was mixed with the loading buffer (Beyotime) and heated in a boiling water bath for 3 min. A 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for protein separation using a SDS-PAGE gel preparation kit (Beyotime). The electrophoresis (1–2 hours) was initiated at 80 V and switched to 120 V after bromophenol blue entered the separation gel. The proteins were transferred to a membrane in an ice bath under a 300 mA current. The transfer of protein was sustained for 60 min. After being rinsed for 1–2 min, the membrane was placed in blocking buffer at room temperature for 60 min or at 4°C overnight. Primary rabbit anti-human GAPDH antibody (internal reference, 5174S, 1:1000, Cell Signaling Technology, Boston, Massachusetts, USA), anti-EphA10 antibody (ab106437, 1–2 µg/mL, Abcam, Cambridge, Massachusetts, USA) and anti-Gli3 antibody (sc-74478, 1:1000, Santa Cruz, Texas, USA) were incubated with the membrane on a shaker at room temperature for 1 hour. The membrane was washed with washing buffer for 3×10 min. The secondary antibody (horseradish peroxidase-labeled goat anti-rabbit IgG, 1:5000, Beijing ComWin Biotech, Beijing, China) was incubated with the membrane at room temperature for 1 hour, followed by another round of membrane washing. The membrane was added with color developing solution, and the protein expression was detected by the chemiluminescence imaging system (Bio-Rad, Hercules, California, USA).

CCK-8 assay

Suspension of transfected MDA-MB-231 or BT20 cells (100 µL) was transferred to a 96-well plate. Each group had three replicate wells. The cells were incubated for 0, 24, 48 and 72 hours before treatment with CCK-8 reagent (10 µL per well, Dojindo, Tokyo, Japan) for 1–4 hours. The absorbance value was measured at 450 nm.

Transwell assay

Transwell inserts (−20°C, Corning, New York, USA) were taken out to thaw the Matrigel at room temperature. The Transwell inserts and 24-well plate were added with 0.5 mL of serum-free medium. The culture fluid was removed after 2 hours. MDA-MB-231 and BT20 cells grown at the logarithmic phase were suspended and cultured in 6-well plates. The cells were transfected as mentioned above after reaching 70%–90% confluence. Each group had three replicate wells. After 24 hours of cultivation, the cells were trypsinized and washed twice with phosphate buffer saline. The cells were resuspended in serum-free DMEM with an adjusted concentration. The basolateral chamber was added with 600 µL of medium containing 10% FBS, and the apical chamber was added with 100 µL of the cell suspension. After 24 hours, the Transwell inserts were taken out and the supernatant was discarded. Cells remaining in the apical chamber were cleaned using a cotton swab. Cells that invaded through the membrane were fixed with 4% paraformaldehyde for 20 min before Wright–Giemsa staining. Number of the cells in five random fields of view was counted under a microscope at high magnification and the cells were photographed.

Scratch assay

MDA-MB-231 and BT20 cells grown at the logarithmic phase were suspended and evenly inoculated in six-well plates. The cells were transfected as stated after 24 hours. A line was vertically scratched in the cell layer using a sterile pipette (100 µL). The scratch width of each group was basically the same. The scratch area at start was recorded as the control. The cells were photographed before and after 24-hour cultivation.

Statistical analysis

SPSS V.17.0 and GraphPad V.7.0 were used. Data are shown in the form of mean±SD. χ^2 test was used for analysis of the relationship between EphA10/Gli3 and clinical factors of BC. Kaplan-Meier and log-rank tests were applied for analysis of the patient survival. A Cox proportional hazards model was established for multivariate analysis. t-test and one-way analysis of variance were used for comparisons between two groups and among multiple groups. Tukey's multiple comparisons test was performed for post hoc multiple comparisons. Significant statistics were represented by $p<0.05$.

RESULTS

Expressions of EphA10 and Gli3 in invasive BC, intraductal BC, pericancerous tissues, lobular hyperplastic tissues and normal breast tissues

Immunohistochemistry showed that EphA10 and Gli3 were located in the cytoplasm (online supplemental figure S1A–B). EphA10 and Gli3 were positively expressed in over 55% of the 124 invasive carcinomas and in over 44% of the 18 intraductal carcinomas (table 3). Positive expressions of EphA10 and Gli3 were found in less than 24% of the 50 pericancerous tissues and 50 lobular hyperplastic tissues (table 3). EphA10 and Gli3 were negatively expressed in all 30 normal breast tissues (table 3). The expressions of EphA10 and Gli3 in invasive carcinomas were significantly higher than those in pericancerous tissues, lobular hyperplastic tissues and normal tissues (table 3, $p<0.01$). Pericancerous tissues and loublar hyperplastic tissues with positive EphA10 and/or Gli3 expression exhibited certain degree of dysplasia. The positive rates of EphA10 and Gli3 were not significantly different in invasive carcinomas and intraductal carcinomas (table 3, $p>0.05$).

Table 3 Expressions of EphA10 and Gli3 in invasive BC, intraductal BC, peritumoral tissues, lobular hyperplastic tissues and normal breast tissues

Tissue type	Case number	EphA10 positive (%)	Gli3 positive (%)
Invasive carcinoma	124	76 (61.3)	69 (55.6)
Intraductal carcinoma	18	8 (44.4)	9 (50.0)
Pericancerous tissues	50	11 (22.0) *	12 (24.0) *
Lobular hyperplastic tissues	50	10 (20.0) *	10 (20.0) *
Normal breast tissues	30	0 (0.0) *	0 (0.0) *

*p<0.01, compared with invasive carcinoma.
BC, breast cancer.

Comparisons of intraductal and invasive BC

Invasive carcinomas had significantly stronger association with advanced histological grade (grade III), large tumor diameter (>3 cm), negative estrogen receptor (ER)/progesterone receptor (PR) and advanced lymph node metastasis (table 1, $p<0.05$). The expression of EphA10 or Gli3 had no significant difference in intraductal carcinomas and invasive carcinomas (table 1, $p>0.05$).

Expressions of EphA10 and Gli3 are associated with the clinical factors of invasive BC

EphA10 and Gli3 showed higher positive rates in cases with histological grade III, lymph node metastasis and advanced TNM stage (table 4, $p<0.05$). The expressions of EphA10 and Gli3 exhibited no significant association with patient age, menopausal status, tumor diameter, ER, PR and CerbB2 status (table 4, $p>0.05$). Among the 76 cases with positive EphA10 expression, 52 cases also had positive Gli3 expression. Among the 48 cases with negative EphA10 expression, 31 cases also exhibited negative Gli3 expression. The expression of EphA10 was positively correlated with that of Gli3 ($\chi^2=12.984$, $p=0.000$).

Correlation of EphA10/Gli3 expression with patient survival

Ninety-eight patients died within 13 years and patients (26 cases) who survived longer than 13 years were included in the analysis as censored cases. Kaplan-Meier survival analysis showed that the mean survival of patients with invasive BC was linked to the histological grade (grade III), tumor diameter (>3 cm) and advanced tumor metastasis (table 5, $p<0.05$). Positive EphA10 or Gli3 expression was associated with poor survival of patients with BC (table 5, online supplemental figure S2, $p=0.000$). Cox multivariate analysis also showed that the abovementioned factors displayed negative correlations with the overall survival of patients with BC. Additionally, both EphA10 and Gli3 acted independently in the prognosis of BC (table 6, $p<0.05$). Finally, we calculated the area under receiver operating

Table 4 Expressions of EphA10 and Gli3 are associated with the clinicopathological characteristics (CPC) of invasive BC

CPC	Case number	EphA10			Gli3		
		Positive number (%)	χ^2	P value	Positive number (%)	χ^2	P value
Age (years)							
≤45	68	43 (63.2)	0.24	0.624	43 (63.2)	3.515	0.061
>45	56	33 (58.9)			26 (46.4)		
Menopausal status							
Premenopausal	71	46 (64.8)	0.857	0.355	45 (63.4)	4.027	0.045
Postmenopausal	53	30 (56.6)			24 (45.3)		
Histologic grades							
I	17	7 (41.2)	12.512	0.002	6 (35.6)	15.254	0.000
II	49	24 (48.9)			20 (40.8)		
III	58	45 (77.6)			43 (74.5)		
Tumor size							
≤3 cm	57	30 (52.6)	3.334	0.068	27 (47.4)	2.928	0.087
>3 cm	67	46 (68.7)			42 (62.7)		
ER							
+	59	30 (50.8)	5.174	0.023	29 (49.2)	1.922	0.166
–	65	46 (70.8)			40 (61.5)		
PR							
+	64	38 (59.4)	0.205	0.651	37 (57.8)	0.252	0.616
–	60	38 (63.3)			32 (53.3)		
CerB2							
+	78	48 (61.5)	0.005	0.941	44 (56.4)	0.05	0.823
–	46	28 (60.9)			25 (54.3)		
Lymph node metastasis							
No	56	27 (48.2)	7.359	0.008	25 (44.6)	5.008	0.025
Yes	68	49 (72.1)			44 (64.7)		
TNM stage							
I+II	66	30 (45.5)	14.914	0.000	23 (34.9)	24.726	0.000
III+IV	58	46 (79.3)			46 (79.3)		

BC, breast cancer; ER, estrogen receptor; PR, progesterone receptor; TNM, tumor, node, metastasis.

characteristic curve (AUC) for EphA10 (AUC=0.706, 95% CI: 0.617 to 0.796) and Gli3 (AUC=0.678, 95% CI: 0.587 to 0.770) (online supplemental figure S3).

EphA10 and Gli3 are highly expressed in invasive BC cells

qRT-PCR and Western blot analysis showed that EphA10 was highly expressed in invasive BC cells (MDA-MB-231, BT20 and Hs578T) compared with normal mammary epithelial cells (MCF10A) (online supplemental figure S4A–B, $p<0.05$). Gli3 was also highly expressed in MDA-MB-231, BT20 and Hs578T cells compared with MCF10A cells (online supplemental figure S4C–D, $p<0.05$).

Knockdown of EphA10 or Gli3 suppresses BC cell proliferation, invasion and migration

MDA-MB-231 and BT20 cells were transfected with sh-EphA10, sh-Gli3, sh-NC or sh-EphA10+sh-Gli3. The expression of EphA10 was significantly decreased in the sh-EphA10 group compared with the sh-NC group (online supplemental figure S5A–B, $p<0.01$). Also, the expression of Gli3 was reduced in the sh-Gli3 group compared with the sh-NC group (online supplemental figure S5C–D, $p<0.01$). The proliferation of MDA-MB-231 and BT20 cells was inhibited in the sh-EphA10 group and sh-Gli3 group compared with the sh-NC group; the cell proliferation was further impeded in the sh-EphA10+sh-Gli3group compared with the sh-Gli3 group (online supplemental figure S5E, $p<0.05$). Transwell and scratch assays revealed that the invasion and migration of MDA-MB-231 and BT20 cells were suppressed in the sh-EphA10 group and sh-Gli3 group compared with the sh-NC group; the cell invasion and migration were further inhibited in the sh-EphA10+sh-Gli3 group compared with the sh-Gli3 group (online supplemental figure S5F–G, $p<0.05$). Taken together, knockdown of EphA10 or Gli3 suppresses the malignant behaviors of BC cells.

DISCUSSION

The synergic effect of EphA10 and Gli3 in BC was first reported in the present study. Overexpresssion of EphA10 and Gli3 was observed in invasive BC tumors and cells. EphA10 and Gli3 expressions were positively associated with the severity of invasive BC. Moreover, EphA10 and Gli3 promoted the malignant behaviors of BC cells in vitro.

Eph receptors, required for normal morphogenesis,[21 22] are aberrantly expressed in diverse human cancers.[23 24] Among the Eph receptors, EphA10 is a kinase-deficient protein.[25] Some preclinical trials have revealed the implication of Eph in tumor growth, metastasis and angiogenesis.[26–28] A variety of cell surface proteins, including Eph receptors, are involved in the progression of BC.[9 29] EphA10 has recently been reported to have correlation with poor prognosis and metastasis of tumors, and high expression of EphA10 has been found in invasive BC cells.[9 13 29–31] Given the deficiency

Table 5 Correlations of clinicopathological characteristics (CPC) and EphA10/Gli3 expression with the mean survival of patients with invasive BC

CPC	Case number	Mean survival (years)	χ^2	P value
Age (years)				
≤45	68	8.22 (0.2–13.0)	0.134	0.714
>45	56	8.99 (0.8–13.0)		
Menopausal status				
Premenopausal	71	7.97 (0.2–13.0)	2.261	0.133
Postmenopausal	53	9.33 (0.8–13.0)		
ER				
+	59	9.75 (0.7–13.0)	1.847	0.174
–	65	7.48 (0.2–13.0)		
PR				
+	64	9.02 (0.7–13.0)	0.019	0.891
–	60	8.05 (0.2–13.0)		
CerB2				
+	78	8.17 (0.7–13.0)	0.688	0.407
–	46	9.17 (0.2–13.0)		
Histologic grades				
I	17	13.51 (11.2–13.0)	61.642	0.000
II	49	11.36 (3.0–13.0)		
III	58	4.70 (0.2–13.0)		
Tumor size				
≤3 cm	57	10.11 (1.0–13.0)	8.604	0.003
>3 cm	67	7.19 (0.2–13.0)		
TNM stage				
I + II	35	18.58 (7–30)	57.569	0.000
III + IV	38	11.05 (3–30)		
Lymph node metastasis				
No	56	10.73 (0.8–13.0)	9.214	0.002
Yes	68	6.77 (0.2–13.0)		
Invasion				
No	33	17.52 (4–30)	17.399	0.000
Yes	67	9.87 (3–30)		
EphA10				
–	48	12.36 (3.0–13.0)	30.874	0.000
+	76	6.12 (0.2–13.0)		
Gli3				
–	55	12.35 (2.0–13.0)	37.617	0.000
+	69	5.69 (0.2–13.0)		

BC, breast cancer; ER, estrogen receptor; PR, progesterone receptor; TNM, tumor, node, metastasis.

Table 6 Multivariate Cox regression analysis of the correlation between EphA10/Gli3 expression and the overall survival of patients with invasive BC

CPC		P value	Relative risk	95% CI Lower	95% CI Upper
Age (years)	≤45/>45	0.029	2.291	1.088	4.823
Menopausal status	Prememopausal /prememopausal	0.061	2.032	0.968	4.265
Tumor size	≤3 cm/>3 cm	0.028	1.859	1.070	3.228
Histologic grade	I/II/III	0.000	3.543	2.213	5.673
Lymph node metastasis	No/yes	0.020	2.521	1.158	5.490
TNM stage	I/II/III/IV	0.004	1.948	1.235	3.074
ER	–/+	0.568	0.797	0.366	1.735
PR	–/+	0.215	0.602	0.269	1.344
CerB2	–/+	0.988	1.004	0.627	1.606
EphA10	–/+	0.001	2.515	1.473	4.293
Gli3	–/+	0.009	1.940	1.180	3.189

BC, breast cancer; CI, confidence interval; CPC, clinicopathological characteristics; ER, estrogen receptor; PR, progesterone receptor; TNM, tumor, node, metastasis.

of kinase activity, the mechanisms for EphA10 activation may explain for its implication in tumorigenesis.

The Hh-related protein Gli3 is implicated in a variety of cancers, but its role in BC remains elusive. A previous study reported that the three Hh signaling proteins, including Gli1, Gli2 and Gli3, were correlated with some prognostic factors in BC and Gli2 expression predicted a lower overall survival rate.[32] Bai *et al*[18] identified Gli3 as a promising prognostic factor in non-small cell lung cancer, and overexpression of Gli3 was crucial for the tumorigenesis. Inactivation of the Gli3 signaling disrupted the interaction between MDM2 and p53 and strongly potentiated p53-dependent inhibition of colon cancer cell growth.[17] The aforementioned studies provide basis for the rational use of Gli3 antagonist as a novel treatment option for cancers.

This study showed that EphA10 and Gli3 were highly expressed in invasive BC tissues and cells. Positive EphA10 or Gli3 expression was correlated with severe clinical situations and poor survival of patients with invasive BC. The AUC of EphA10 and Gli3 showed that these two proteins could be considered as targets for early diagnosis and prevention of BC. In vitro experiments further demonstrated that EphA10 or Gli3 promoted the malignant behaviors of BC cells, and coexpression of EphA10 and Gli3 exerted greater carcinogenic effect.

In conclusion, EphA10 and Gli3 are critical promoters in invasive BC. Positive EphA10 and Gli3 expressions are associated with poor prognosis of invasive BC. Moreover, EphA10 and Gli3 promote the malignant behaviors of BC cells.

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Patient consent for publication Not required.

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Data availability statement Data are available in a public, open access repository. The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

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