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ORIGINAL ARTICLE

Combining bioinformatics analysis and experiments to explore *CARNS1* as a prognostic biomarker for breast cancer

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Abstract

Background: Breast cancer is a heterogeneous malignant disease, which has variation in clinical behaviors. High-throughput technologies have added important genetic alternative and biological change information for breast cancer. *CARNS1* is an important ATPases. It can catalyze the synthesis of carnosine, which has antiproliferative activity in cancer. Here, we hypothesize that *CARNS1* plays an essential role in the development of breast cancer.

Methods: The expressions of *CARNS1* in breast cancer were data-mined and analyzed from TCGA (the Cancer Genome Atlas) and GEO (the Gene Expression Omnibus) databases. The correlation of *CARNS1* expression with clinical characteristics and the diagnostic capability of *CARNS1* were assessed. Experimental studies were conducted in two cohorts ($n = 60$) of breast cancer patients by qRT-PCR and immunohistochemical analysis.

Results: *CARNS1* was significantly downregulated in breast cancer. The expression was correlated with tumor molecular and histological types, T and M stages, and vital status. Kaplan–Meier survival analysis showed that the downregulation of *CARNS1* was significantly related to poor overall survival and relapse-free survival. Moreover, these scenarios have been extended to ER, PR, and HER2 positive patients. Univariate and multivariate analysis showed that *CARNS1* can be considered as an independent prognostic predictor for patients with breast cancer. Experimental data supported that the protein and mRNA levels of *CARNS1* in breast cancer are indeed significantly downregulated.

Conclusion: Our findings have demonstrated that *CARNS1* acts as a tumor suppressor gene and may be an independent prognostic indicator for breast cancer patients.

KEYWORDS
breast cancer, *CARNS1*, data mining, prognosis

1 | INTRODUCTION

Breast cancer is a heterogeneous malignant tumor. Worldwide, it is the most commonly diagnosed cancer among women (Ferlay et al., 2015). Over the last decades, despite the molecular characteristics of breast cancer have largely impacted in the treatment decisions, and multimodal therapies have improved the chances of cure for 70–80% of early-stage patients (Harbeck et al., 2019), advanced breast cancer (metastasis and invasion) remains the most common cause

of death (Scully et al., 2012). Therefore, finding a prognostic biomarker that can classify patients into different treatment categories may help for management of the disease.

Carnosine is originally discovered in skeletal muscle and is a naturally occurring dipeptide-containing histidine that is highly concentrated in the brain and muscle tissues (Boldyrev et al., 2013). It can be used as an intracellular pH buffer and antioxidant (Artioli et al., 2019), and has many applications in health and disease, including healthy aging, improved cognitive function, and prevention of diabetes complications (Artioli et al., 2019; Hipkiss & Chana, 1998). It has been shown that supplementation with β-alanine can increase the level of carnosine in muscle (Sale et al., 2013). As a result, high levels of carnosine may improve athletic performance and ability (Artioli et al., 2010; Baguet et al., 2010), suggesting the potential therapeutic effect of carnosine. Recent studies have also expanded their physiological roles in the brain, heart, pancreas, and kidneys. In particular, carnosine exposure has been shown to have a positive effect on reducing the growth of human glioblastoma cells (Renner et al., 2010), intestinal tract (Shen et al., 2014), and ovarian cells (Mikula-Pietrasik & Ksiazek, 2016). Carnosine synthase 1 (*CARNS1*) (OMIM #613368) was identified as an ATP-grasp domain-containing protein 1, which is mainly distributed in the skeletal muscle and central nervous system of vertebrates (Drozak et al., 2010). *CARNS1* catalyzes the synthesis of carnosine and homocarnosine (Kwiatkowski et al., 2018). Gene Ontology (GO) annotations indicate that the biosynthesis and histidine metabolic pathways of homocarnosine may be related to the biological functions of *CARNS1* (Veiga-da-Cunha et al., 2014). However, the functions of carnosine and *CARNS1* are still largely unknown, especially their involvement in carcinogenesis.

In the current study, we elucidated the different expression of *CARNS1* in breast cancer patients by exploring the Cancer Genome Atlas database (ACGT) and verified the gene expression in four Gene Expression Omnibus (GEO) and experimental samples. The potential clinical significance of *CARNS1* was also assessed.

2 | MATERIALS AND METHODS

2.1 | Ethical compliance

The study was approved by the Medical Ethics Committee of the Second Hospital of Jilin University, and all patients have consented for the study.

2.2 | Data source

Public high-throughput RNA-sequencing data were acquired from the Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/). Four groups of gene microarrays with survival data (GSE22035, GSE31138, GSE40057, and GSE92252) were retrieved from the Gene Expression Omnibus (GEO) of National Center of Biotechnology Information (NCBI) database (Clough & Barrett, 2016). All data were downloaded on 20 August 2019.

2.3 | Data mining and statistical analyses

RTCGA toolbox package (Release 3.10) (Obadia et al., 2012) was used to explore the clinical characteristics. We first performed ROC analysis to evaluate the diagnostic ability of *CARNS1* (NC_000011.10). Based on the ROC curve, the optimal cutoff value was determined and was further used to dichotomize patients into low and high *CARNS1* expression groups (Kadara et al., 2011). In order to distinguish the expression differences in various variables, a boxplot was used. The chi-squared and Fisher's exact tests were utilized to evaluate the association between *CARNS1* expression and clinical variables. Kaplan–Meier method was used to generate survival data, and the results were compared by log-rank test. First, the data were analyzed using the univariate model. If the prognosis is meaningful, the Cox proportional hazards regression model is used to further analyze the data. In all analyses, a statistically significant was defined as a p-value less than 0.05.

2.4 | Experimental validation of *CARNS1* expression

Forty-four formalin-fixed and paraffin-embedded surgical samples from woman diagnosed with invasive ductal or lobular breast cancer and 10 adjacent normal breast tissues were collected. The expression of *CARNS1* at mRNA level was measured by quantitative real-time PCR (qRT-PCR) method. Total RNA was extracted using Recover All Total Nucleic Acid Isolation kit (Ambion). According to the manufacturer's recommendation, reverse transcription of RNA was used Super Script III reverse transcriptase (Invitrogen Corp.). The primers were designed with Primer 3 and the forward primer was 3′-tttgcatcccagttggtaca-5′ and the reverse primer was 3′-tgaggctcttctgcttagcc-5′. qRT-PCR analyses were performed using SYBR Green Master Mix (Applied Biosystems) and the ABI PRISM 7900 Sequence Detection System (Applied Biosystems Inc., CA, USA). The 2-ΔΔCt method was used to calculate the relative fold change of mRNA expression, in which the average of ΔCt values of the target amplicon was normalized to the endogenous gene (GAPDH), compared with 10 normal breast tissue specimens. Each experiment was repeated three times.

Additional custom-designed breast cancer TMA was used to evaluate *CARNS1* protein expression. The TMA contains 16

TABLE 1 Clinical characteristics of patients

Characteristics	No. (%)
Age	
<60	589 (53.45)
>=60	513 (46.55)
Histological-type	
No record	3 (0.27)
Infiltrating ductal carcinoma	790 (71.56)
Infiltrating lobular carcinoma	204 (18.48)
Other	107 (9.69)
Molecular-subtype	
No record	255 (23.1)
Basal	142 (12.86)
HER2	67 (6.07)
LumA	422 (38.22)
LumB	194 (17.57)
Normal	24 (2.17)
T-classification	
No record	2 (0.18)
T1/2	921 (83.42)
T3/4	178 (16.12)
TX	3 (0.27)
N-classification	
No record	2 (0.18)
N0/1	883 (79.98)
N2/3	199 (18.03)
NX	20 (1.81)
M-classification	
No record	2 (0.18)
M0	917 (83.06)
M1	22 (1.99)
MX	163 (14.76)
Stage	
No record	10 (0.91)
I/II	808 (73.19)
III/IV/X	286 (25.91)
Lymph-node-status	
No record	379 (34.33)
No	28 (2.54)
Yes	697 (63.13)
Vital-status	
No record	2 (0.18)
Deceased	155 (14.04)
Living	947 (85.78)
Radiation_therapy	
No record	102 (9.24)
No	445 (40.31)
Yes	557 (50.45)
Neoadjuvant_treatment	
No record	3 (0.27)
No	1088 (98.55
Yes	13 (1.18)
Targeted_molecular_therapy	
No record	525 (47.55)
No	46 (4.17)
Yes	533 (48.28)
CARNS1	
High	681 (61.68)
Low	423 (38.32)

(Continues)

TABLE 1 (Continued)

tumors and paired adjacent normal samples in the FFPE tissue blocks. The expression of *CARNS1* was determined by immunohistochemistry using a rabbit polyclonal antibody (HPA038569, Sigma-Aldrich). Briefly, the TMA was cut to 4-μm section, deparaffined and rehydrated. The antigen was retrieved by using citrate acid buffer in a microwave oven. Endogenous peroxidase was treated with 3% of hydrogen peroxide and nonspecific binding was blocked by normal goat serum. The primary rabbit anti-*CARNS1* (1:100) antibody was then applied on the section overnight at 4°C. The Universal LSAB^{TM}2 detective system (DakoCytomation) was used to detect positive signal. Hematoxylin was used as a counterstain agent.

3 | RESULTS

3.1 | Patient population

The *CARNS1* expression and clinical characteristics data from total of 1102 breast cancer patient were obtained from TCGA database. The detailed clinical variables, including age, histologic/molecular types, stage, TNM classifications, lymph node status, vital status, and *CARNS1* expression are shown in Table 1.

3.2 | Downregulated *CARNS1* expression in TCGA breast cancer data set

To analyze the expression of *CARNS1* in the TCGA data set, boxplots were utilized. As shown in Figure 1a, by compared with normal tissue samples, the expression level of *CARNS1* in breast cancer samples was significantly lower ($p = 8.8e{-}10$). There were significantly different expressions

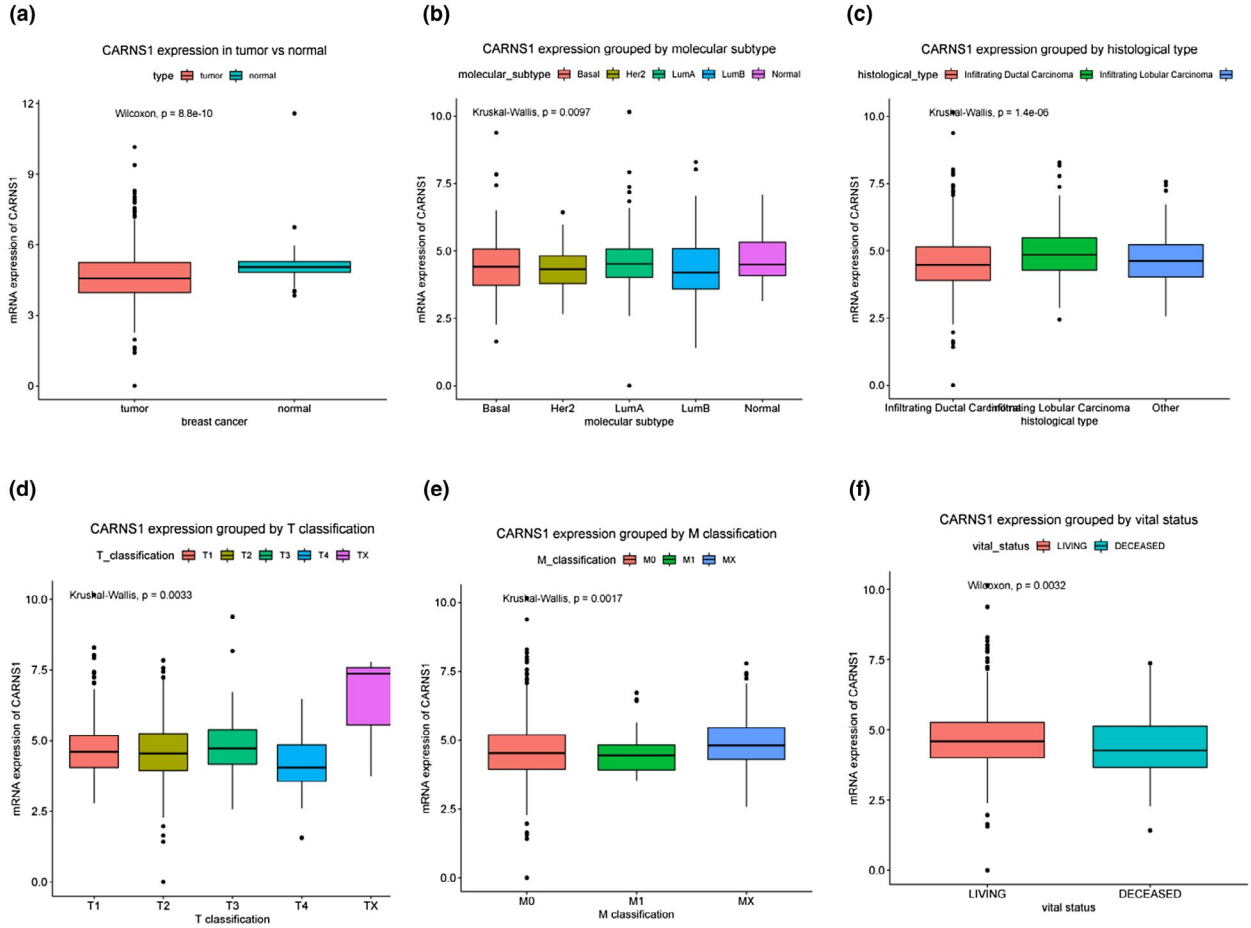

FIGURE 1 The differential expression of *CARNS1* in breast cancer patients. Boxplot showed in groups by (a) tumor versus normal; (b) molecular type; (c) histological type; (d) T classification; (e) M classification; and (f) vital status. All $p \leq 0.003$

of *CARNS1* in groups by molecular subtype ($p = 0.0097$, Figure 1b), histological type ($p = 1.4e{-}06$, Figure 1c), T and M stage ($p = 0.0033$ and $p = 0.0017$, respectively. Figure 1d,e), and vital status ($p = 0.0032$, Figure 1f). However, no significant changes were observed for groups by age, margin, menopause statuses, various therapies, stage, and N classification (Figure S1a–h). ROC curve analyses (Figure 2) indicated that *CARNS1* had a moderate diagnostic capability with an AUC of 0.674 (Figure 2a). Further stage analysis consistently showed diagnostic capability, in that the AUC ranged from 0.666 to 0.703 (Figure 2b–e).

3.3 | Clinical significance of *CARNS1* expression in breast cancer

In order to further study the relationship between *CARNS1* expression and patient survival, the *CARNS1* expression value was used to divide the patient cohort into high and low groups based on the ROC curve analysis. The analysis results are depicted in Table 2. The expression of *CARNS1* was significantly correlated with age, ER, menopause status, TNM classifications, stage, and vital status. Notable, *CARNS1* expression was also correlated with radiation therapy.

3.4 | Expression of *CARNS1* was associated with overall and relapse-free survivals in breast cancer

Kaplan–Meier survival analysis demonstrated that patients with low *CARNS1* expression levels associated with worse overall survival (OS) (Figure 3a, $p < 0.0001$) and worse relapse-free survival (RFS) (Figure 4a, $p = 0.0021$), compared to those with high *CARNS1* expression patients. Subgroup analysis suggested that low *CARNS1* expression group had a worse OS in patients with histological types of infiltrating ductal and lobular ($p = 0.0036$ and $p = 0.039$; Figure 3b,c), or in the ER, PR, and HER2 positive groups ($p = 0.001$, $p = 0.026$, and $p = 0.009$; Figure 3d–f). Similarly, a worse RFS was associated with decreased *CARNS1* expression in infiltrating ductal cancer ($p = 0.0034$, Figure 4b), ER, PR, and HER2 positive patients ($p = 0.011$, $p = 0.015$, and $p = 0.0019$; Figure 4d–f).

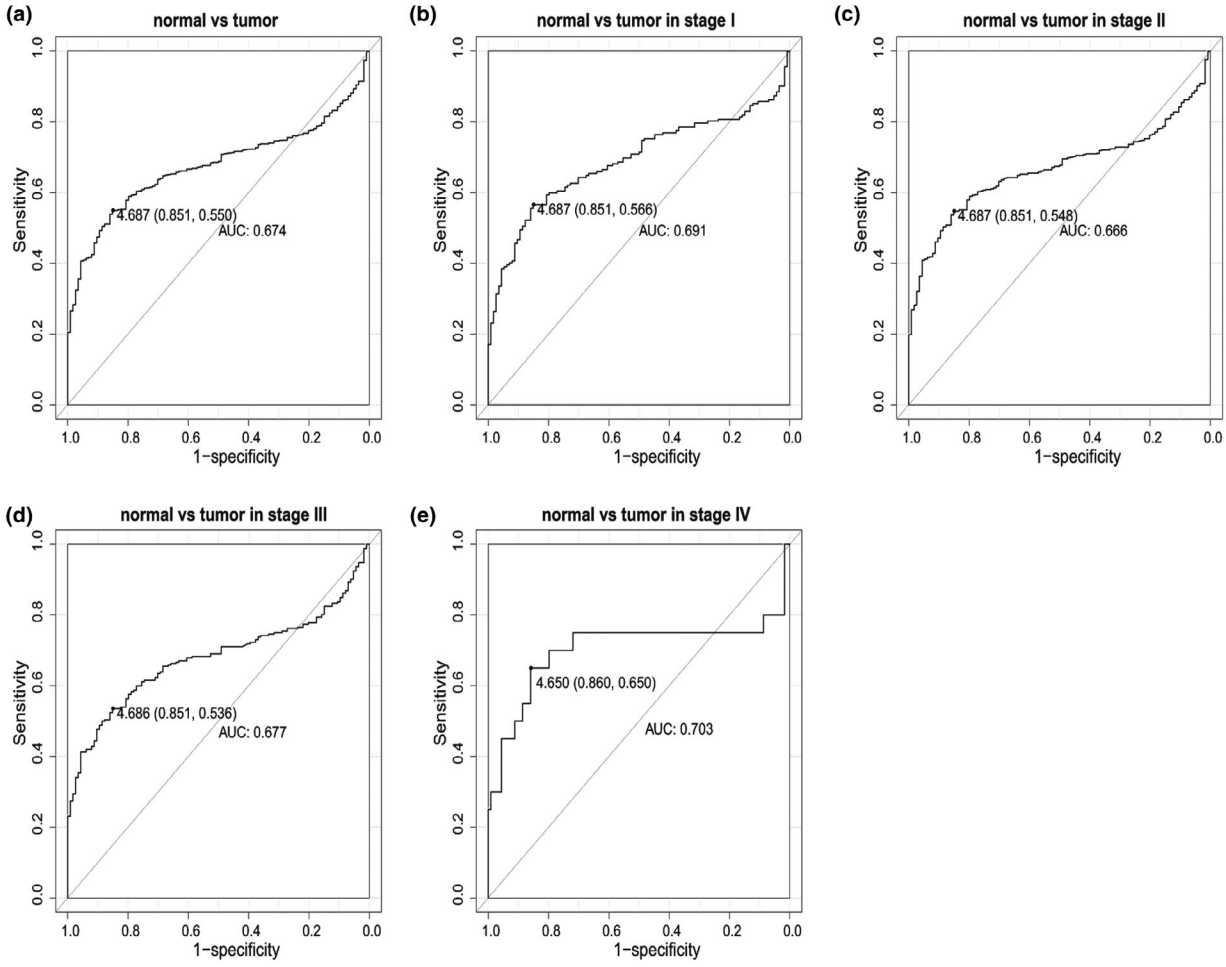

FIGURE 2 ROC curve analysis of *CARNS1* diagnostic capability in breast cancer. (a) Normal versus tumor; (b) normal versus tumor stage I; (c) normal versus tumor stage II; (d) normal versus tumor stage III; (e) normal versus tumor stage IV

Univariate analysis demonstrated that age, HER2 status, stage, resection margin status, and *CARNS1* expression were variables related to OS (all $p < 0.01$; Table 3). Multivariate analysis indicated that for OS, the downregulation of *CARNS1* was a self-dependent prognostic factor ($p = 0.005$, Table 3). A similar analysis was performed on RFS, and the lower expression of *CARNS1* remained a self-dependent prognostic factor for RFS ($p = 0.009$, Table 4).

3.5 | Validation of downregulated *CARNS1* expression in GEO data set

Four breast cancer-related GEO databases (GSE22035, GSE31138, GSE40057, and GSE92252) were downloaded and assessed. As shown in Figure S2, two microarrays (GSE22035, Figure S2a, and GSE92252, Figure S2d) demonstrated that *CARNS1* expression in breast cancer tissues was significantly lower than that in noncancerous tissues. $p = 0.038$ and $p = 0.024$, respectively. GSE31138 (Figure S2b) showed a borderline change between cancer and normal groups. Although there was no significant difference between cancer and normal tissues in the GSE40057 (Figure S2c) data set, relatively low-expression levels of *CARNS1* were observed in the cancer group, indicating that the overall expression of *CARNS1* was decreased in breast cancer tissues as compared to noncancerous tissue.

3.6 | Experimental validating *CARNS1* expression in cancer patient samples

The expression of *CARNS1* transcriptional mRNA level was evaluated by qRT-PCR in 44 available breast cancer patient samples. By comparing with 10 normal tissues, the expression level of *CARNS1* in most cases was lower in the cancer group (Figure 5a, $p = 0.01$). The expression of *CARNS1* was further determined in an independent cohort of 16 pairs of cancer and normal TMA breast cancer samples using immunohistochemistry. Two of the 16 cases showed weak cytoplasm/membranous expression of *CARNS1*, and the remaining samples were negative (Figure 5b). Overall, our

TABLE 2 Correlation of *CARNS1* expression with clinicopathological variables

			CARNS1 expression					
Parameters	**Variables**	***N***	**High**	**%**	**Low**	**%**	***χ2***	***p*-Value**
Age	<60	589	70	(45.16)	519	(54.8)	4.5983	0.032
	>=60	513	85	(54.84)	428	(45.2)		
Histological_type	Infiltrating ductal carcinoma	790	110	(70.97)	680	(71.88)	5.0585	0.0797
	Infiltrating lobular carcinoma	204	23	(14.84)	181	(19.13)		
	Other	107	22	(14.19)	85	(8.99)		
Molecular_subtype	Basal	142	1	(50)	20	(15.62)	7.0442	0.5319
	Her2	67	0	(0)	14	(10.94)		
	LumA	422	1	(50)	56	(43.75)		
	LumB	194	0	(0)	32	(25)		
	Normal	24	0	(0)	6	(4.69)		
ER	Indeterminate	2	2	(1.37)	0	(0)	16.3736	0.0003
	Negative	239	42	(28.77)	197	(21.7)		
	Positive	813	102	(69.86)	711	(78.3)		
PR	Indeterminate	4	1	(0.68)	3	(0.33)	3.5526	0.1693
	Negative	345	57	(39.04)	288	(31.75)		
	Positive	704	88	(60.27)	616	(67.92)		
HER2	Equivocal	180	16	(16.33)	164	(19.93)	4.008	0.2606
	Indeterminate	12	0	(0)	12	(1.46)		
	Negative	565	59	(60.2)	506	(61.48)		
	Positive	164	23	(23.47)	141	(17.13)		
Menopause_status	Inde	34	15	(11.81)	19	(2.15)	37.7846	0
	Peri	40	1	(0.79)	39	(4.41)		
	Post	706	91	(71.65)	615	(69.57)		
	Pre	231	20	(15.75)	211	(23.87)		
T_classification	T1	281	33	(21.29)	248	(26.19)	23.2734	0.0001
	T2	640	81	(52.26)	559	(59.03)		
	T3	138	25	(16.13)	113	(11.93)		
	T4	40	15	(9.68)	25	(2.64)		
	TX	3	1	(0.65)	2	(0.21)		
N_classification	N0	516	44	(28.39)	472	(49.84)	47.1318	0
	N1	367	63	(40.65)	304	(32.1)		
	N2	120	22	(14.19)	98	(10.35)		
	N3	79	15	(9.68)	64	(6.76)		
	NX	20	11	(7.1)	9	(0.95)		
M_classification	M0	917	124	(80)	793	(83.74)	76.9858	0
	M1	22	17	(10.97)	5	(0.53)		
	MX	163	14	(9.03)	149	(15.73)		
Stage	I	182	16	(10.46)	166	(17.64)	88.3012	0
	II	626	70	(45.75)	556	(59.09)		
	III	252	45	(29.41)	207	(22)		
	IV	20	15	(9.8)	5	(0.53)		
	X	14	7	(4.58)	7	(0.74)		
Lymph_node_status	No	28	5	(4.95)	23	(3.69)	0.1113	0.7387
	Yes	697	96	(95.05)	601	(96.31)		

TABLE 2 Continued

Parameters	Variables	*N*	*CARNS1* expression				$\chi 2$	*p*-Value
			High	%	Low	%		
Vital_status	Deceased	155	155	(100)	0	(0)	1093.742	0
	Living	947	0	(0)	947	(100)		
Radiation_therapy	No	445	60	(54.05)	385	(43.21)	4.2726	0.0387
	Yes	557	51	(45.95)	506	(56.79)		
Neoadjuvant_treatment	No	1088	152	(98.06)	936	(98.94)	0.2887	0.591
	Yes	13	3	(1.94)	10	(1.06)		
Targeted_molecular_therapy	No	46	10	(14.29)	36	(7.07)	3.447	0.0634
	Yes	533	60	(85.71)	473	(92.93)		
Overall survival	0	933	0	(0)	933	(100)	1078.792	0
	1	154	154	(100)	0	(0)		
Relapse-free survival	0	816	41	(44.09)	775	(94.63)	221.1809	0
	1	96	52	(55.91)	44	(5.37)		

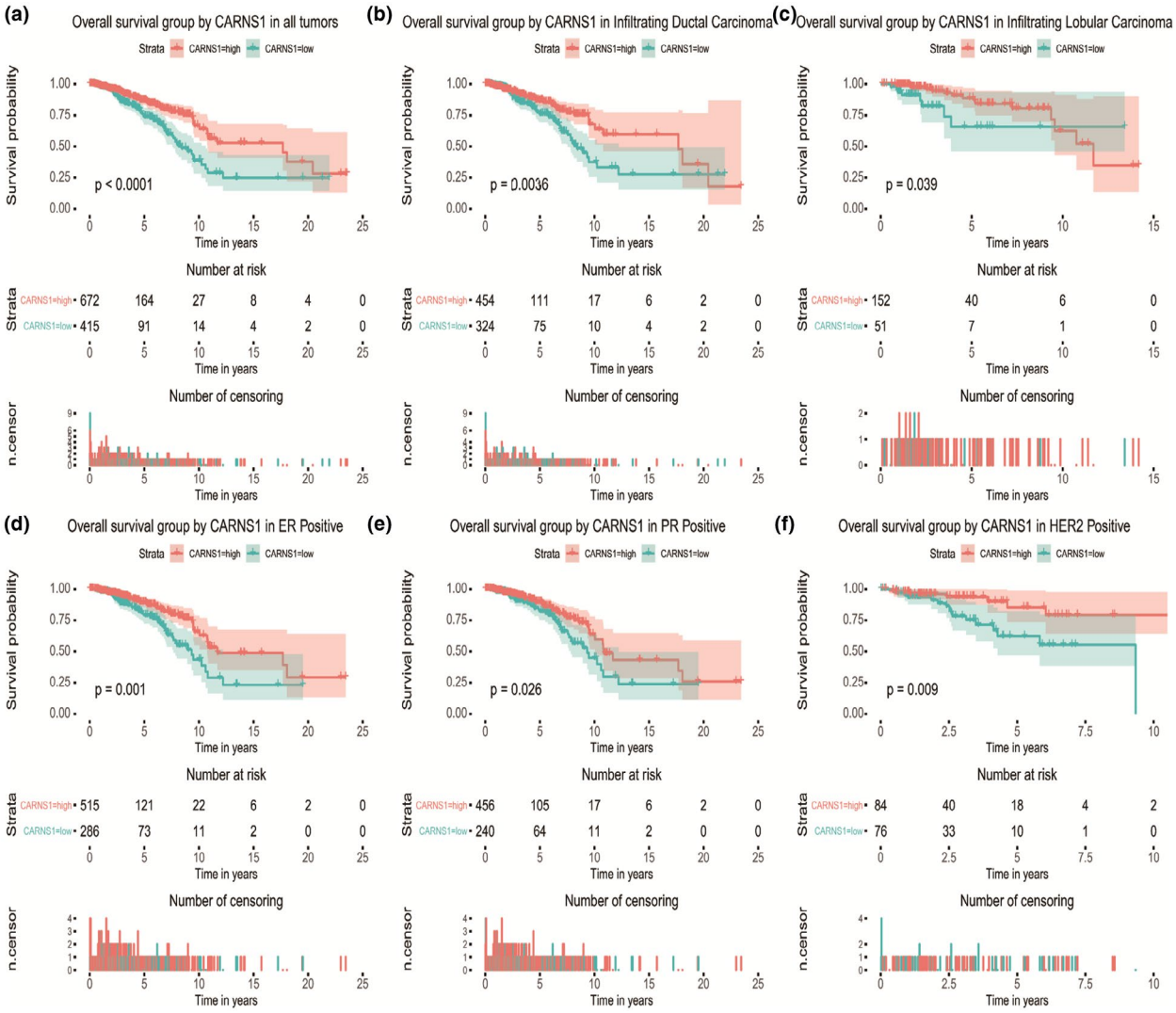

FIGURE 3 Overall survival analysis of *CARNS1* expression. Kaplan–Meier curves produced in (a) all tumors; (b) infiltrating ductal carcinomas; (c) infiltrating lobular carcinomas; (d) ER positive; (e) PR positive; and (f) HER2 positive. All $p \leq 0.05$

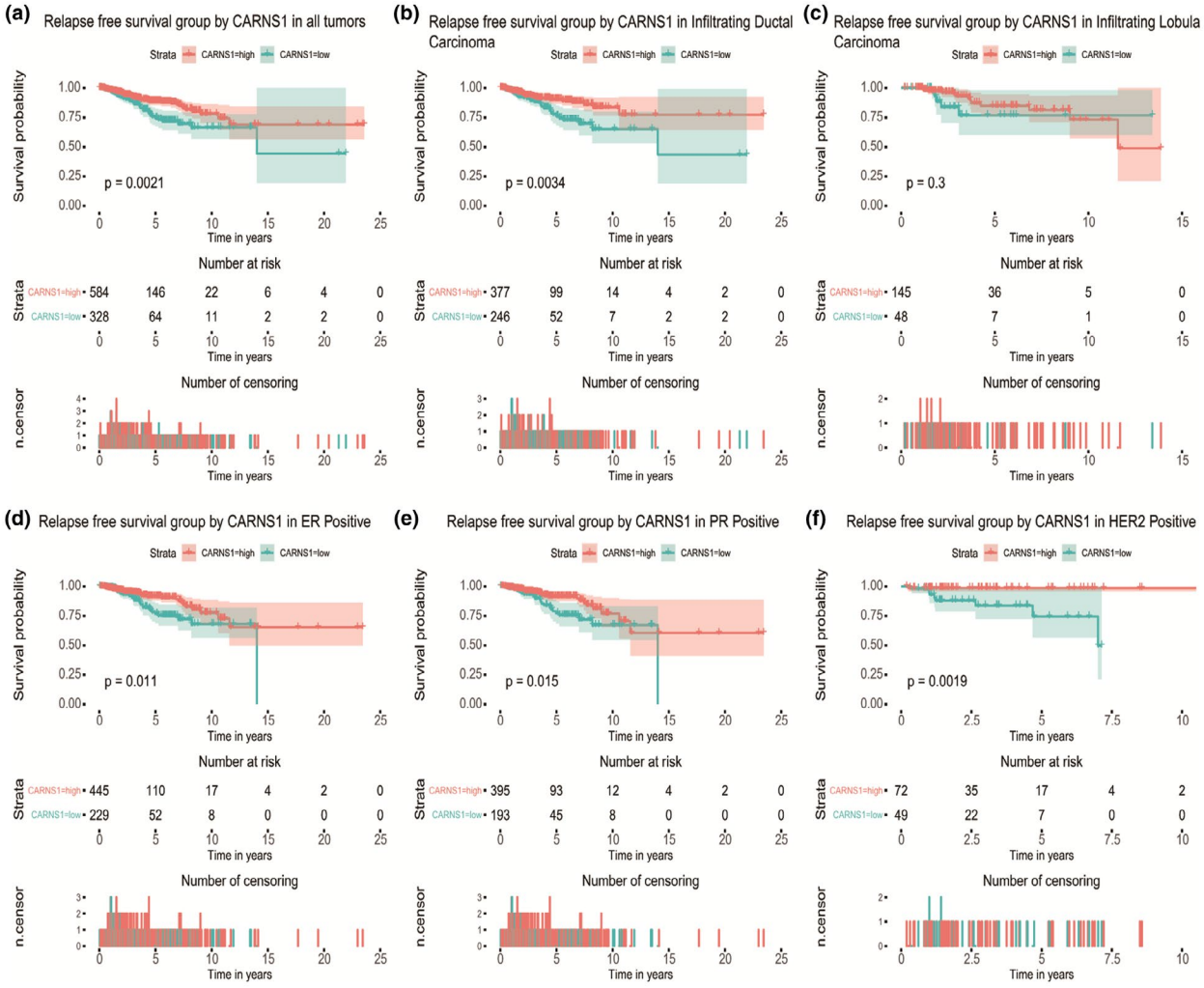

FIGURE 4 Relapse-free survival analysis of *CARNS1* expression. Kaplan–Meier curves produced in (a) all tumors; (b) infiltrating ductal carcinomas; (c) infiltrating lobular carcinomas; (d) ER positive; (e) PR positive; and (f) HER2 positive. a, b and d–f, $p \leq 0.01$

results demonstrated that *CARNS1* was indeed significantly downregulated in breast cancer at both protein and mRNA levels.

4 | DISCUSSION

Based on the TCGA breast cancer data set ($n = 1102$) and the analysis of four GEO databases, we demonstrated that *CARNS1* was significantly downregulated in breast cancer. Interestingly, the aberrant expression of *CARNS1* associated significantly with poor overall survival and poor relapse-free survival. In addition, experimental data provided evidence that *CARNS1* was downregulated in the two cohorts of breast cancer patients, indicating that *CARNS1* plays an important role as an independent prognostic predictor of breast cancer patients. To the extent of our knowledge, this is the first study to explore the crucial role of *CARNS1* in carcinogenesis.

Most previous studies have focused on carnosine. Increasing evidence has implicated the potential applications of carnosine in health and disease, especially its antiproliferative properties. Carnosine suppresses glycolytic energy metabolism in human malignancy, which is critical for cancer cells (Renner et al., 2010). Injection of carnosine can inhibit the sympathetic nerve activity of the rat spleen and reduce the proliferation of rat colon cancer cell (Horii et al., 2012). It has also been suggested that carnosine treatment can reduce the proliferation of gastric carcinoma cells through Akt/mTOR signaling activation, cell cycle arrest, and increases apoptosis (Zhang et al., 2014). Despite these encouraging findings, the expression of carnosine and its related peptides or its synthases, such as *CARNS1* in cancer tissues has not been explored yet. *CARNS1* is considered to be a tumor suppressor gene silenced in breast cancer, and only one previous study has been reported in breast cancer cell lines.

In the current study, we found that *CARNS1* is downregulated in breast cancer, which is coordinated with the antiproliferative properties of carnosine in various cancer cells (Figure 1a). This observation was further validated in two other cohorts of breast cancer patients at Gene Expression

TABLE 3 Univariate and multivariate analysis for overall survival in breast cancer patients

	Univariate analysis			Multivariate analysis		
Parameters	**Hazard ratio**	**CI 95**	***p*-Value**	**Hazard ratio**	**CI 95**	***p*-Value**
Age	1.91	1.39–2.63	0	2.25	1.40–3.59	0.001
Histological type	0.93	0.74–1.17	0.543			
Molecular subtype	1.01	0.88–1.16	0.901			
ER	0.85	0.71–1.02	0.074			
PR	0.87	0.73–1.03	0.096			
HER2	1.29	1.05–1.57	0.013	1.14	0.91–1.41	0.248
Menopause status	1.16	0.94–1.43	0.165			
Stage	1.64	1.40–1.91	0	2.04	1.54–2.71	0
Lymph node status	1.1	0.93–1.30	0.274			
Margin status	1.42	1.11–1.81	0.005	0.95	0.68–1.34	0.781
CARNS1	1.92	1.40–2.64	0	1.93	1.22–3.06	0.005

TABLE 4 Univariate and multivariate analysis for relapse-free survival in breast cancer patients

	Univariate analysis			Multivariate analysis		
Parameters	**Hazard ratio**	**CI 95**	***p*-Value**	**Hazard ratio**	**CI 95**	***p*-Value**
Age	1.45	0.97–2.16	0.072			
Histological type	0.86	0.65–1.14	0.29			
Molecular subtype	0.99	0.88–1.16	0.901			
ER	0.78	0.63–0.97	0.026	0.84	0.6–1.17	0.294
PR	0.78	0.64–0.96	0.019	0.87	0.64–1.19	0.392
HER2	0.93	0.7–1.22	0.596			
Menopause status	0.95	0.74–1.22	0.713			
Stage	1.71	1 1.4–2.08	0	1.54	1.23–1.91	0
Lymph node status	0.86	0.7–1.06	0.159			
Margin status	1.59	1.23–2.06	0	1.5	1.14–1.97	0.004
CARNS1	1.86	1.25–2.78	0.002	1.76	1.15–2.69	0.009

Omnibus (Figure S2a,d). Subgroup analysis showed that the expression of *CARNS1* has significant differences in regard to molecular subtypes, histological types, T and M classifications, and vital status (Figure 1b–f). Notably, the expression of *CARNS1* was considerably higher in Tx (tumor cannot be measured) (Figure 1d) and Mx (metastasis cannot be measured) (Figure 1e) than the other subgroups, indicating that *CARNS1* may be correlated to tumor progression. Although the downregulation of *CARNS1* has been observed in our own patient cohort, their relative clinical information still has limitations, so a larger cohort sample may be needed for further study.

Because of the heterogeneity of breast cancer, the clinical course will vary between patients. Next-generation sequencing and gene profiling studies have distinguished different molecular subtypes of breast cancer, such as luminal A, luminal B, HER2 enriched, and basal like based on the six-marker expression subtype panel of ER, PR, HER2, CK5/6, EGFR, and Ki-67 (Liu et al., 2015; Voduc et al., 2010). Extensive studies have demonstrated that different molecular subtypes of breast tumors are related to different clinical behaviors. Moreover, these molecular classifications play a guiding role in treatment management (Coleman, 2017; Taherian-Fard et al., 2015). In our study, we found that different expressions of *CARNS1* are associated with different ER, PR, and HER2 status. *CARNS1* was significantly correlated with worse OS in ER positive group only, while in the PR and HER2 positive or negative groups, *CARNS1* expression was associated with poor OS. Likewise, an analysis was performed to assess relapse-free survival. Regardless of whether ER, PR, or HER2 is positive or negative, the low expression of *CARNS1* is related to poor RFS. However, regarding OS and RFS, no significant correlation was observed between *CARNS1* expression and different molecular subtypes, which indicates that *CARNS1* may play

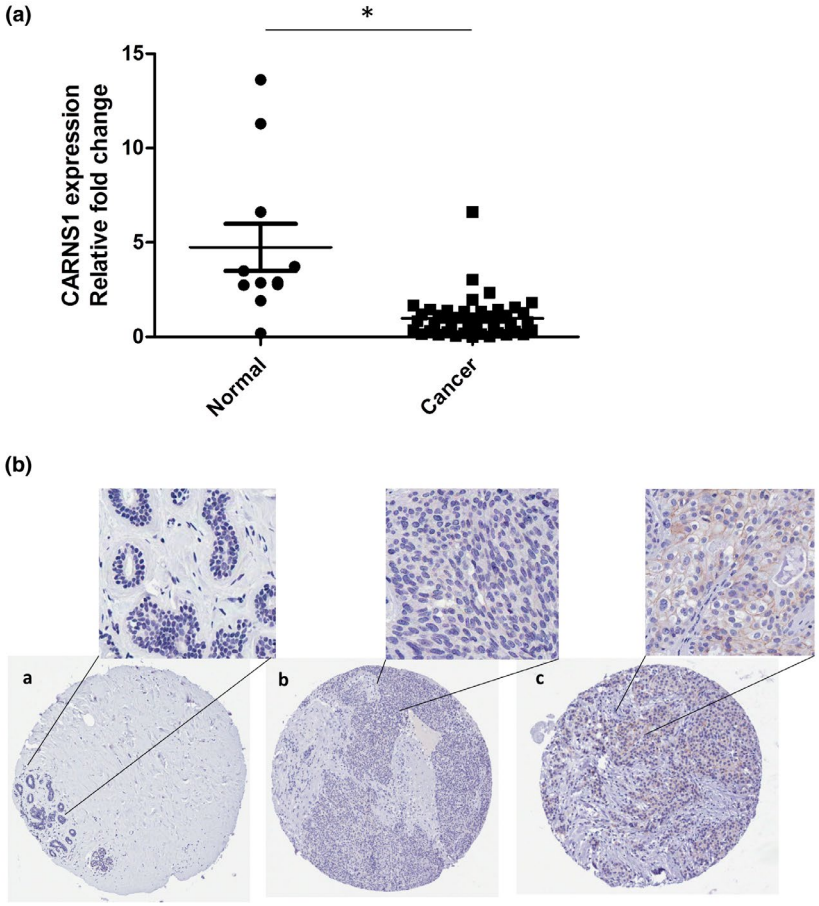

FIGURE 5 Downregulation of *CARNS1* in breast cancer patients. (a) *CARNS1* expression in 44 breast cancer patient samples and 10 normal breast epithelial samples. *CARNS1* was measured using qRT-PCR. $*p = 0.01$. (b) Immunohistochemical staining of *CARNS1* in an independent set of 16 breast cancer TMA. a. Representative image showing no expression of *CARNS1* in normal breast tissues. (b) Representative image for negative expression of *CARNS1* in tumor cells. (c) Representative image showing weak cytoplasmic and membrane expression of *CARNS1* in tumor cells. (200× and 400× magnifications)

different roles or mechanisms in patients with different ER, PR, and HER2 status. Of course, further investigation is required to study its underlying mechanism.

In conclusion, we reported that *CARNS1* is downregulated in breast cancer patients. The low expression of *CARNS1* is significantly correlated with the poor overall survival and relapse-free survival in breast cancer patients. Furthermore, *CARNS1* expression is associated with several breast cancer molecular markers, which will provide new opportunities for the prognosis and treatment of this heterogeneous disease.

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CONFLICT OF INTEREST

The authors report that no conflict of interest presents in this work.

AUTHOR CONTRIBUTIONS

SxQ and MH designed and led the study concept; LZ, YZ, and XZ retrieved data and conducted analysis; XL contributed to the investigation; LZ, MH, and SxQ contributed to the writing of the manuscript. All authors have read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data sets that were used and/or analyzed in this study are available from the corresponding author upon request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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