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Original Paper

The Clinicopathological Significance and Correlative Signaling Pathways of an Autophagy-Related Gene, Ambra1, in Breast Cancer: a Study of 25 Microarray RNA-Seq Datasets and in-House Gene Silencing

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Key Words
Activating molecule in Beclin-1-regulated autophagy (Ambra1) • Breast cancer • Microarray • RNA sequencing • Knock-down

Abstract
Background/Aims: The activating molecule in Beclin1-regulated autophagy (Ambra1) has been observed to be over-expressed in several cancers, but the clinical contribution of Ambra1 in breast cancer (BC) remains unknown. Hence, in this study, we conducted a comprehensive investigation into the expression, biological role, and underlying functional mechanism of Ambra1 in BC. ***Methods:*** Microarray and RNA-seq datasets providing Ambra1 expression data were obtained from Gene Expression Omnibus (GEO), ArrayExpress, Oncomine, and The Cancer Genome Atlas (TCGA). Both standard mean deviation (SMD) and summary receiver operating characteristic methods were employed to assess Ambra1 expression in BC. We then silenced Ambra1 in MDA-MB-231 cells and performed *in vitro* experiments to explore the biological effects of Ambra1 on BC cells. Furthermore, differentially expressed genes (DEGs) after Ambra1 knock-down were profiled with a microarray and overlapped with the genes correlated with Ambra1 from Multi Experiment Matrix (MEM) and genes similar to Ambra1 from Gene Expression Profiling Interactive Analysis. These overlapping genes were collected for further bioinformatics analyses to investigate the underlying molecular mechanism of Ambra1 in BC. ***Results:*** A total of 25 microarray and RNA-seq datasets involving 2460 breast cancer samples were included. The pooled results demonstrated that Ambra1 was markedly up-regulated in BC tissues (SMD=0.39, 95% CI=0.15–0.63; P=0.002), and the Ambra1 level

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was also significantly related to the progression of BC, especially metastasis status (P=0.004). *In vitro* experiments suggested that the proliferation of MDA-MB-231 cells transfected with Ambra1 short hairpin RNA (sh-RNA 2450) showed a decreasing trend at 48 h compared with the control (CK) group. However, apoptosis was similar in cells transfected with Ambra1 sh-RNAs and in the CK cells. Furthermore, we performed a microarray-based comparison of genes after Ambra1 knock-down. The 828 DEGs from microarray analysis were intersected with 4266 Ambra1 co-expressed genes from MEM. Eventually, the overlapped 183 genes were found to be enriched in several well-known cancer-related pathways, including the MAPK signaling pathway, chronic myeloid leukemia pathway, and VEGF signaling pathway. ***Conclusion:*** These results indicate that the level of Ambra1 up-regulation is clearly related to tumorigenesis and progression of BC, probably via influencing several vital pathways. However, this hypothesis needs to be validated with more in-depth experiments in the future.

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Introduction

Autophagy, an essential eukaryotic process of self-digestion, is associated with the lysosomal degradation of cytosolic components and can occur in physiological or pathological circumstances. Of interest, autophagy can serve contradictory roles in tumor promotion and suppression. A number of crucial proteins are involved in the process of autophagy, including BECN1/Beclin 1 and activating molecule in Beclin1-regulated autophagy (Ambra1), which is a positive regulator of Beclin1 [1-3]. Ambra1, a WD40 domain-containing protein, has been documented to be involved in the growth and progression of the central nervous system, adult neurogenesis, and vertebrate embryogenesis [4]. Ambra1 can be adversely modulated by mTOR phosphorylation, which inhibits binding to the E3-ligase TRAF6 and ULK1 ubiquitylation, therefore modulating the stability and function of ULK1 [5, 6]. Moreover, Ambra1 is able to bind c-Myc to the phosphatase PP2A, which finally leads to c-Myc degradation, decreased cell growth, and carcinogenesis [7-9].

Concurrently, Ambra1 has been observed to be over-expressed in several cancers, including pancreatic ductal adenocarcinoma (PDAC) [10], cholangiocarcinoma [11], prostate cancer [12], cutaneous melanoma [13], and gastric adenocarcinoma [14]. Furthermore, the clinicopathological effect of Ambra1 has also been reported in several malignancies. Accumulation of Ambra1 appeared to be related to the status of lymph node metastasis and worse survival of cholangiocarcinoma patients [11]. Up-regulated Ambra1 was also positively associated with the Gleason score in prostate cancer [12]. Ambra1 was further identified to be a new independent prognostic marker for early stage melanomas [13]. Most recently, a high level of Ambra1 was found to be significantly correlated with depth of invasion and lymph node metastasis in gastric adenocarcinoma (GC) [14]. More importantly, an increase in Ambra1 level could be an independent predictor of worse survival in GC patients. These findings suggest that the occurrence of autophagy via Ambra1 could be associated with a malignant phenotype and progression in several malignancies.

Breast cancer (BC) is the most common malignancy among females worldwide, and is a heterogeneous and multiplex disease with multiple pathogenic factors [15-17]. The 5-year survival of BC will be greatly improved if the disease is diagnosed at an early stage, yet the outcome of 15% of BC patients is still poor due to diagnosis at an advanced stage [18-22]. Based on estrogen and progesterone receptors, human epidermal growth factor receptor type 2 (HER2) and Ki-67, BC patients have been categorized into different subtypes, including luminal A, luminal B, Her2 over-expressing (Her+), and triple negative breast cancer subtypes, which are sensitive to various treatment regimens [23-26]. Nevertheless, treatment algorithms do not definitely give rise to favorable clinical outcomes. Consequently, sensitive and accurate predictive biomarkers are needed to improve prognosis and treatment efficacy [27-30].

The functions and mechanisms involved in autophagy in BC cells have been extensively investigated [31-33]. However, understanding of the prospective role of Ambra1 in BC remains limited. Only one study conducted by Shen et al [34]. showed that cisplatin induced autophagy in BC cells and up-regulated multiple autophagy-related genes, including Ambra1. Hence, in the present study, we conducted a comprehensive investigation of Ambra1 expression based on high-throughput data from different sources, including the Gene Expression Omnibus (GEO), ArrayExpress, Oncomine, and The Cancer Genome Atlas (TCGA). We then silenced Ambra1 in MDA-MB-231 cells and performed *in vitro* experiments to explore the biological effects of Ambra1 on BC cells. Furthermore, the differentially expressed genes (DEGs) after Ambra1 knock-down were profiled with a microarray and overlapped with the Ambra1-correlated genes from Multi Experiment Matrix (MEM) and genes similar to Ambra1 from Gene Expression Profiling Interactive Analysis (GEPIA). These overlapped genes were collected for further bioinformatics analyses to investigate the underlying molecular mechanism of Ambra1 in BC.

Materials and Methods

Studies based on data from GEO, ArrayExpress, Oncomine, and TCGA: Data downloading and processing from GEO, ArrayExpress, Oncomine, and TCGA

BC-related mRNA microarray datasets were collected from GEO (http://www.ncbi.nlm.nih.gov/geo/), ArrayExpress (http://www.ebi.ac.uk/arrayexpress/), and Oncomine (https: //www. oncomine.org/resource/main.html) up to January 6, 2018. Search strategy was ((breast OR mammary OR mastocarcinoma) AND (cancer OR carcinoma OR tumor OR neoplas* OR malignan*) AND (mRNA OR gene OR protein)). Additionally, we retrieved Ambra1-related RNA-seq datasets and corresponding clinical and survival data from TCGA (http://cancergenome.nih.gov/) to evaluate the clinical significance of Ambra1 in BC.

The following inclusion criteria were applied to select eligible datasets: (1) human research; (2) both BC samples and normal controls were included in each dataset; (3) each group comprised no less than three samples; and (4) Ambra1 expression data in each group were provided.

Essential information such as data source, platform, first author, publication year, sample size, and Ambra1 expression level in both cancer and normal control groups was collected by two reviewers independently (Rong-quan He and Dan-dan Xiong). In the case of disagreement, these reviewers would consult with a third investigator (Gang Chen).

Statistical analysis

All Ambra1 expression data were log2-transformed. The expression pattern and diagnostic capability of Ambra1 in each included dataset were visualized in the form of scatter plots and receiver operating characteristic (ROC) diagrams using GraphPad Prism 5 (La Jolla, CA) and SPSS 20.0 (IBM, Armonk, NY). Next, we integrated all individual datasets. A combined standard mean deviation (SMD) was calculated and a summary receiver operating characteristic (SROC) curve was generated with STATA 12.0 (Stata Corporation, College Station, TX). The chi-squared-based Cochrane Q-test and I-square (I^2) test were used to test underlying heterogeneity among the included datasets. Values of $P>0.05$ and/or $I^2<50\%$ indicated no heterogeneity, and thus we selected a fixed-effects model; otherwise, we chose a random-effects model. Begg's and Egger's tests were used to detect potential publication bias. $P>0.05$ represented no significant publication bias. The relationships between Ambra1 expression and both clinicopathological parameters and survival outcomes in patients with BC were evaluated based on clinical information from TCGA. Student's t-test or one-way analysis of variance were utilized to compare the associations between Ambra1 expression and clinical characteristics, such as age, gender, ER, PR, HER2, TNM stage, T stage, N stage, M stage, and molecular subtype. Kaplan–Meier survival analysis and log rank test were applied to evaluate the prognostic value of Ambra1 in patients with BC. $P<0.05$ was considered to indicate statistical significance.

In vitro experiments: Down-regulation of Ambra1 using short hairpin RNA (shRNA) in BC MDA-MB-231 cells

MDA-MB-231 cells (TCHu227; Cell bank, Chinese Academy of Sciences) were cultured at 37°C in RPMI-1640 supplemented with 10% fetal calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humid incubator with 5% CO_2. Specific-target Ambra1 sh-RNA (2450) and control scrambled plasmids were synthesized by GenePharma (China). The sequence of 2450 was GCT GGA ATC TTC CCT CAT TTC. The plasmids were transfected into MDA-MB-231 cells using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA). To assay the knock-down effects on Ambra1, the proteins were detected using Western blotting after transfection with the plasmids for 48 h. Rabbit polyclonal anti-Ambra1 (sc-130116) was obtained from Santa Cruz Biotechnology, Inc., Dallas, TX. Goat polyclonal anti-rabbit GAPDH (MAB5465) was purchased from MultiSciences (Lianke) Biotech Co., Ltd., Hangzhou, China.

Cell proliferation and apoptosis

Cells were seeded at 8×10^3 cells per well in 96-well flat-bottomed plates (Corning, Corning, NY) and were allowed to attach overnight at 37°C. Then, appropriate medium was added to each well and cells were further cultured at 37°C for the indicated times. The numbers of viable and apoptotic cells were estimated using the cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Absorbance was measured at 450 nm with a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA). Results are from three independent experiments for each group. Statistical analysis was performed using SPSS 22.0 (IBM, Armonk, NY). Significance between groups was analyzed via independent sample *t*-test. $P<0.05$ was considered statistically significant.

DEGs after Ambra1 knock-down in BC MDA-MB-231 cells

BC MDA-MB-231 cells with and without Ambra1 knock-down were used for microarray detection with OneArray (http://www.OneArray.com, PhalanxBio, Inc. San Diego, CA). All mRNAs were annotated according to NCBI RefSeq release 70 and Ensembl release 80 cDNA sequences. Array data were processed using Limma (Bioconductor). The DEGs were required to meet the following criteria: log2 |Fold change| ≥ 1 and $P<0.05$.

Bioinformatics analysis

Collection of genes co-expressed with and similar to Ambra1. Genes co-expressed with Ambra1 were collected from MEM (http://biit.cs.ut.ee/mem/index.cgi). Genes similar to Ambra1 were collected from GEPIA (http://gepia.cancer-pku.cn/).

Functional annotation and protein-protein interaction (PPI) network construction. To further explore the underlying molecular mechanism of Ambra1 in BC, we collected overlapping genes from co-expressed genes and DEGs for further Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. We also constructed a PPI network based on these overlapping genes using the STRING (http://www.string-db.org/). In addition, we obtained overlapping genes from co-expressed genes, DEGs, and similar genes for further gene set enrichment analysis using the GSEA (http://software.broadinstitute.org/gsea/msigdb/collections.jsp#H) database.

Results

In this study, we conducted a comprehensive analysis of the potential role of Ambra1 in BC. The overall design of this study is shown in Fig. 1.

Expression level of Ambra1 in BC

A total of 25 microarray and RNA-seq datasets involving 2460 BC samples and 531 normal controls were included in our study, and information from these eligible studies is presented in Table 1. The expression levels of Ambra1 in individual datasets are displayed as scatter plots and ROC curves (Fig. 2-3). Since the results from individual microarrays were not consistent, a comprehensive analysis was needed. The SMD and corresponding

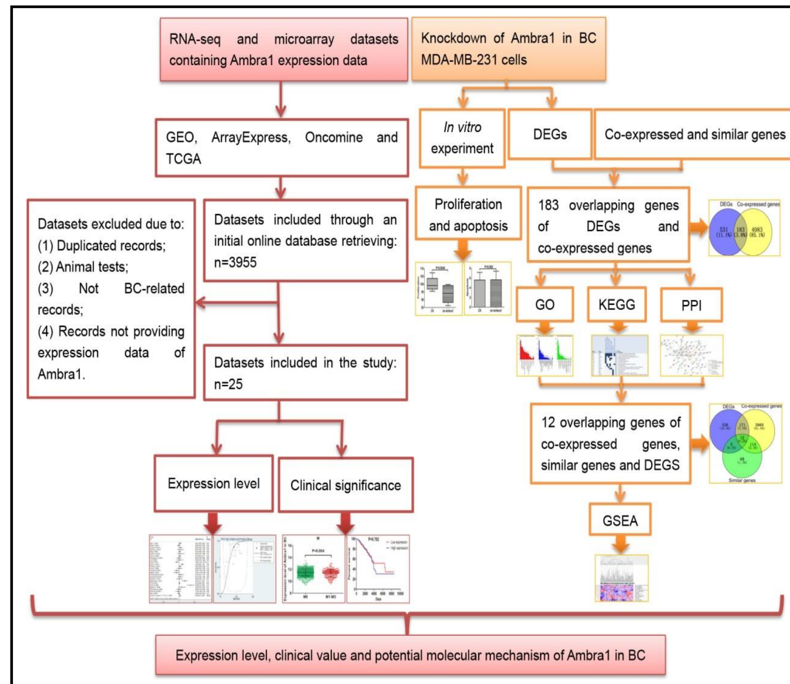

Fig. 1. Study design flow chart. BC: breast cancer; GEO: Gene Expression Omnibus; TCGA: The Cancer Genome Atlas; DEGs: differentially expressed genes; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; PPI: protein–protein interaction; GSEA: Gene Set Enrichment Analysis.

95% confidence interval (CI) were calculated to estimate the pooled expression level of Ambra1 in BC. A random-effects model was applied as inter-study heterogeneity was found (I^2=71.1%, P<0.0001). The combined SMD indicated that the expression level of Ambra1 in BC samples was higher than in normal controls (SMD=0.39, 95% CI: 0.15–0.63; P=0.002; Fig. 4A). Begg's and Egger's tests showed no publication bias (Begg's P=0.097, Egger's P=0.345; Fig. 4B). Then, SROC curve analysis was conducted to evaluate the ability of Ambra1 in differentiating BC patients from normal controls. The area under the curve was 0.80 (95% CI: 0.76–0.83; Fig. 4C), with diagnostic sensitivity and specificity of 0.65 (95% CI: 0.54–0.75) and 0.80 (95% CI: 0.68–0.88), respectively. Additionally, as shown in Fig. 5, the high expression of Ambra1 protein in BC tissues was validated using data from The Human Protein Atlas (https://www.proteinatlas.org/).

Table 1. Essential information on the 25 included datasets. GEO: Gene Expression Omnibus; TCGA: The Cancer Genome Atlas; SD: standard deviation; BC: breast cancer

Data source	Platform	First author (publication year)	Region	Sample size		Mean±SD	
				BC	Normal	BC	Normal
GEO: GSE54002	GPL570	Miki Y (2014)	Singapore	417	16	3.90±0.13	3.84±0.06
GEO: GSE61723	GPL16686	Mathe A (2015)	Australia	48	17	2.67±0.36	2.67±0.29
GEO: GSE29431	GPL570	Cuadros M (2011)	Spain	54	12	3.26±0.23	3.12±0.18
GEO: GSE22544	GPL570	Hawthorn L (2010)	USA	16	4	2.71±0.53	2.38±0.57
GEO: GSE15852	GPL96	Bong I (2009)	Malaysia	43	43	7.62±0.99	7.29±1.0
GEO: GSE25407	GPL570	Latimer JJ (2010)	USA	5	5	5.74±0.53	5.47±0.15
GEO: GSE10780	GPL570	Chen DT (2009)	USA	42	143	3.67±0.18	3.62±0.12
GEO: GSE7904	GPL570	Richardson AL (2007)	USA	43	7	6.45±0.48	6.44±0.15
GEO: GSE6883	GPL96 GPL97	Liu R (2007)	USA	6	6	9.22±0.19	9.15±0.21
GEO: GSE65194	GPL570	Dubois (2015)	France	153	11	8.02±0.26	8.18±0.20
GEO: GSE42568	GPL570	Clarke C (2013)	Ireland	104	17	7.61±0.58	6.94±0.30
GEO: GSE10810	GPL570	Pedraza V (2011)	Spain	31	27	7.56±0.27	7.46±0.18
GEO: GSE10797	GPL571	Casey T (2008)	USA	28	5	3.85±0.45	3.73±0.48
GEO: GSE5764	GPL570	Turashvili G (2007)	Czech Republic	10	20	6.57±0.99	7.15±0.79
GEO: GSE61724	GPL6244	Mathe A (2015)	Australia	64	4	6.71±0.18	6.85±0.24
GEO: GSE61304	GPL570	Yenamandra SP (2015)	Singapore	58	4	6.38±0.19	6.24±0.08
GEO: GSE33692	GPL5175	Knudsen ES (2012)	USA	39	6	8.59±0.46	8.30±1.08
GEO: GSE21422	GPL570	Schaefer C (2011)	Germany	15	4	7.94±0.21	7.90±0.13
GEO: GSE36295	GPL6244	Manikandan J (2011)	Saudi Arabia	45	5	6.96±0.26	6.44±0.23
TCGA	None	TCGA (2017)	USA	1091	113	11.46±0.60	11.31±0.49
Oncomine	None	Finak G (2008)	Canada	31	6	12.29±2.19	4.17±0.25
Oncomine	None	Radvanyi L (2005)	USA	32	3	2.82±0.71	2.04±1.06
Oncomine	GPL570	Richardson AL (2006)	USA	40	7	6.70±0.51	6.69±0.11
Oncomine	GPL570	Karnoub AE (2007)	USA	7	15	7.92±0.23	7.97±0.27
Oncomine	GPL1352	Ma XJ (2009)	USA	38	28	4.76±0.60	4.76±0.56

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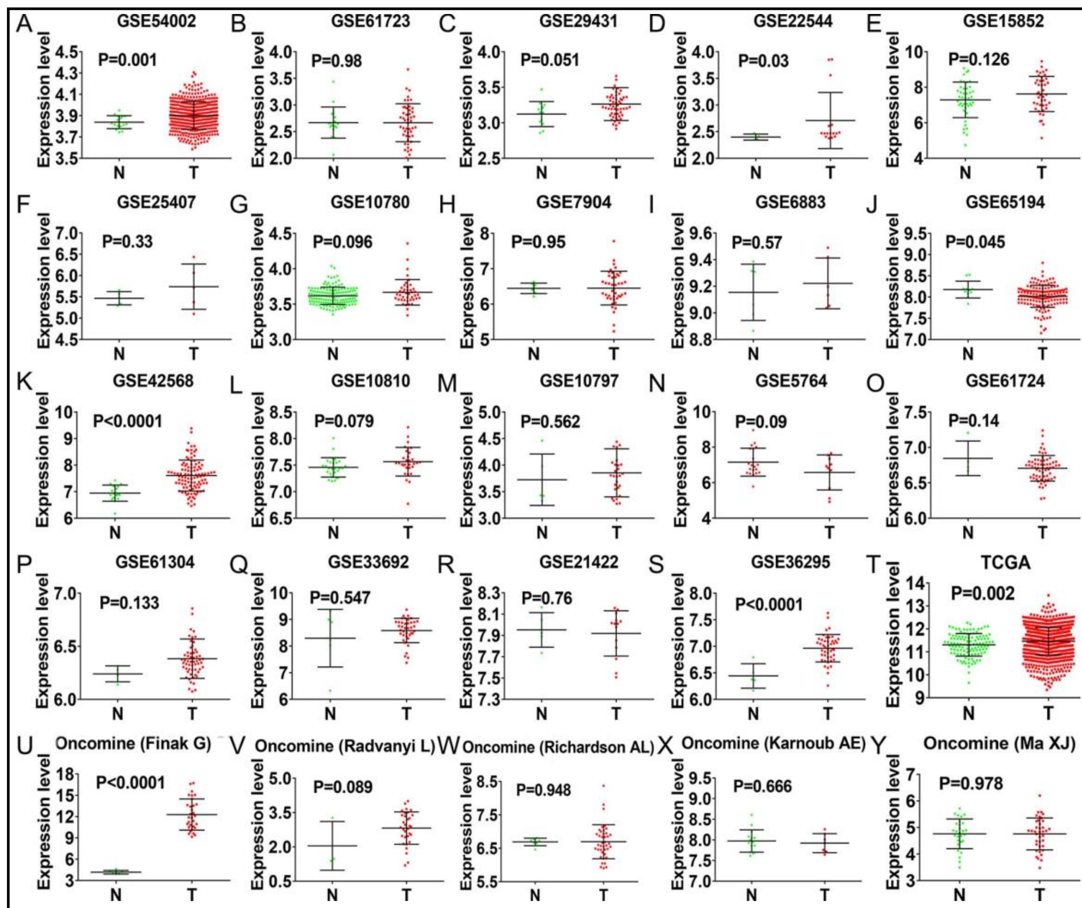

Fig. 2. Scatter plots evaluating the expression level of Ambra1 in breast cancer (BC) (A) GSE54002, (B) GSE61723, (C) GSE29431, (D) GSE22544, (E) GSE15852, (F) GSE25407, (G) GSE10780, (H) GSE7904, (I) GSE6883, (J) GSE65194, (K) GSE42568, (L) GSE10870, (M) GSE10797, (N) GSE5764, (O) GSE61724, (P) GSE61304, (Q) GSE33692, (R) GSE21422, (S) GSE36295, (T) TCGA, (U) Oncomine (Finak G), (V) Oncomine (Radvanyi L), (W) Oncomine (Richardson AL), (X) Oncomine (Karnoub AE), (Y) Oncomine (Ma XJ). N: normal breast samples; T: BC samples.

Clinical significance and prognostic value of Ambra1 in BC

Relationships between Ambra1 expression and clinicopathological parameters are shown in Table 2. We found that Ambra1 expression was significantly correlated with age (P=0.023; Fig. 6A), estrogen receptor (ER) status (P<0.0001; Fig. 6B), progesterone receptor (PR) status (P=0.001; Fig. 6C), M stage (P=0.004; Fig. 6D), and molecular subtype (Fig. 6E). However, no clear correlations were found between Ambra1 expression and gender, HER2 status, TNM stage, T stage, and N stage.

The prognostic role of Ambra1 in BC was explored based on data from the TCGA and Kaplan–Meier plot (http://kmplot.com/analysis). However, the results showed no statistically significant relationship between Ambra1 expression and survival outcome in patients with BC (Fig. 6F-6I).

Additionally, alteration of Ambra1 in BC was explored using data from cBioPortal (http://www.cbioportal.org/), a public database that collects and processes data from TCGA. We found that the alteration rate of Ambra1 in BC was 1.6% (Fig. 6J).

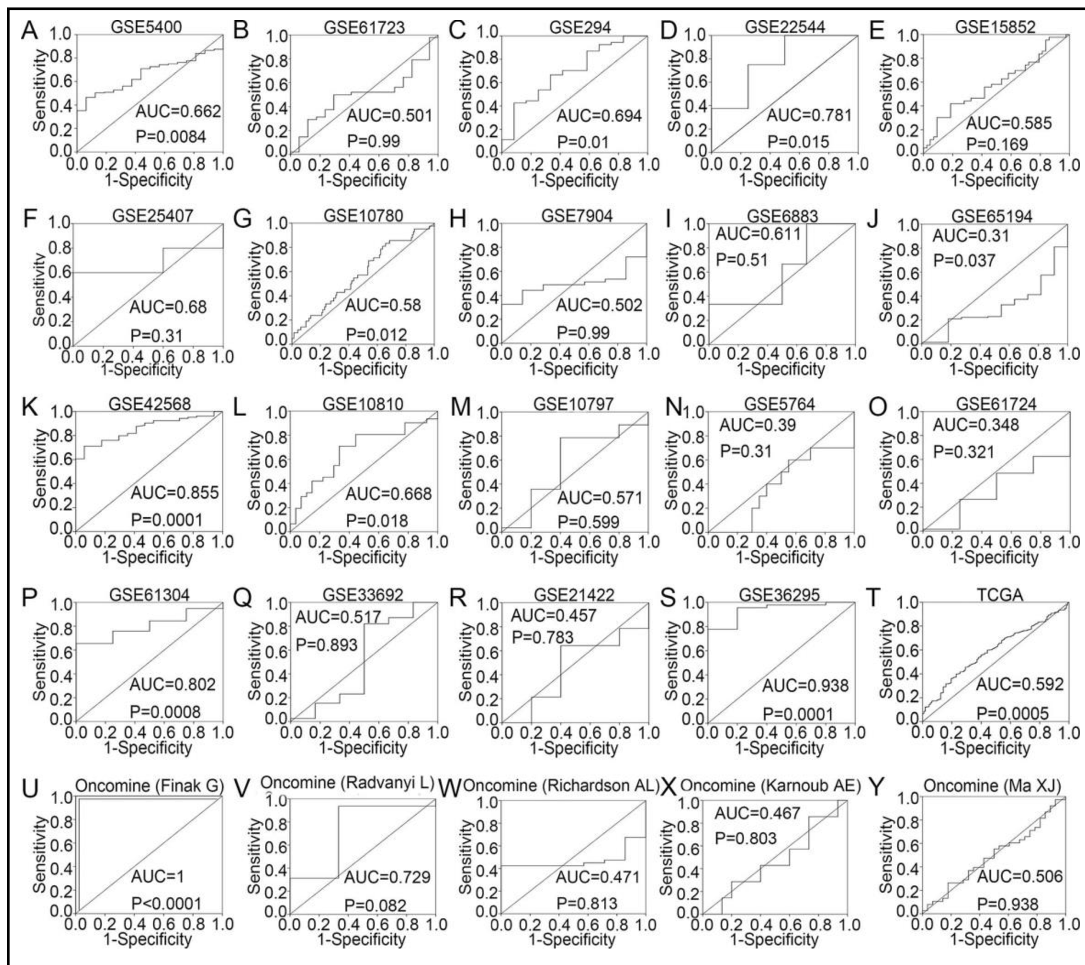

Fig. 3. Receiver operating characteristic curves for the ability of Ambra1 to differentiate breast cancer patients from normal controls. (A) GSE54002, (B) GSE61723, (C) GSE29431, (D) GSE22544, (E) GSE15852, (F) GSE25407, (G) GSE10780, (H) GSE7904, (I) GSE6883, (J) GSE65194, (K) GSE42568, (L) GSE10870, (M) GSE10797, (N) GSE5764, (O) GSE61724, (P) GSE61304, (Q) GSE33692, (R) GSE21422, (S) GSE36295, (T) TCGA, (U) Oncomine (Finak G), (V) Oncomine (Radvanyi L), (W) Oncomine (Richardson AL), (X) Oncomine (Karnoub AE), (Y) Oncomine (Ma XJ). AUC: area under the curve.

Effects of Ambra1 on cell proliferation and apoptosis

The target-specific Ambra1 sh-RNA 2450 was transfected into MDA-MB-231 cells; the knock-down efficiency was 60% as detected by Western blotting (Fig. 6K). Viable and apoptotic cells were examined using CCK-8 (Dojindo Molecular Technologies, Inc.). The proliferation of MDA-MB-231 cells transfected with Ambra1 sh-RNA 2450 showed a decreasing trend at 48 h compared with the CK group (Fig. 6L). Apoptosis was similar in cells transfected with Ambra1 sh-RNAs and CK cells (Fig. 6M).

Collection of DEGs and co-expressed genes

DEGs after Ambra1 knock-down were analyzed using microarray and 828 genes were found, comprising 71 up-regulated and 757 down-regulated genes (Supplementary Fig. S1A-1B - for all supplemental material see www.karger.com/10.1159/000495483). Genes that were co-expressed with Ambra1 were downloaded from the MEM database, and four probes (234237_S_AT, 52731_AT, 234236_AT, and 219141_S_AT) were applied (Supplementary Fig. S2-5). Genes that were detected in at least two probes were considered co-expressed with Ambra1. A total of 4266 co-expressed genes were selected for further analysis.

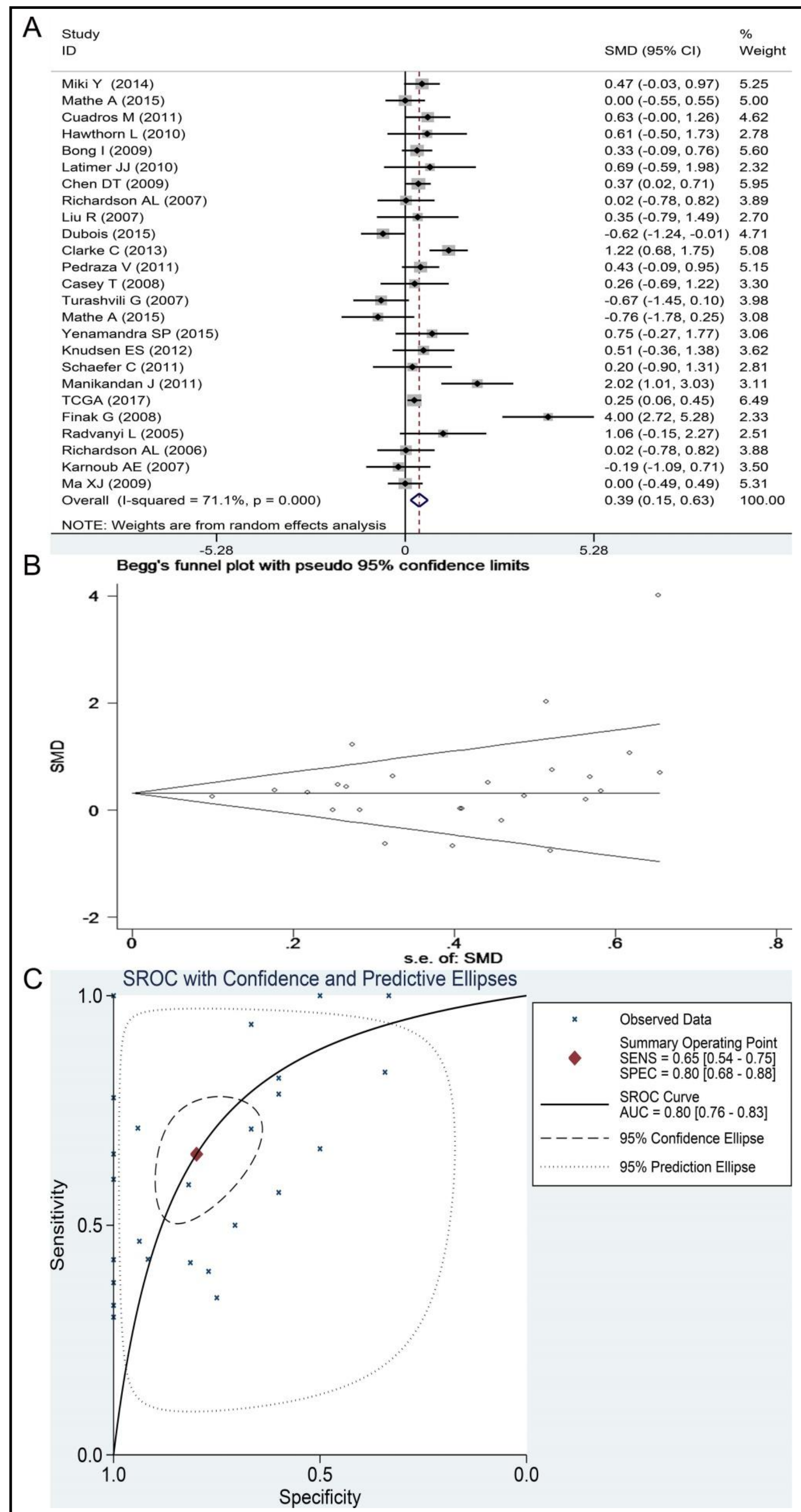

Fig. 4. Expression level of Ambra1 in breast cancer (BC). (A) Forest plot of the 25 datasets evaluating Ambra1 expression in BC (random-effects model). SMD >0 indicates that Ambra1 was up-regulated in BC samples compared with normal controls. (B) Funnel plot evaluating the potential publication bias. (C) SROC curve assessing the ability of Ambra1 to differentiate BC patients from normal controls. SMD: standard mean deviation; SROC: summary receiver operating characteristic; AUC: area under the value.

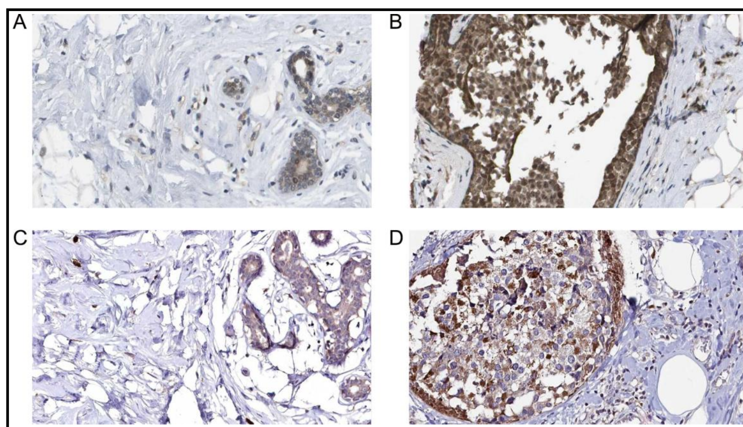

Fig. 5. Immunohistochemical staining of Ambra1 protein based on The Human Protein Atlas database (https://www.proteinatlas.org/). (A) Low expression of Ambra1 protein in normal breast tissues based on antibody CAB17825. (B) High expression of Ambra1 protein in BC tissues based on antibody CAB17825. (C) Low expression of Ambra1 protein in normal breast tissues based on antibody HPA038535. (D) High expression of Ambra1 protein in BC tissues based on antibody HPA038535. Magnification: ×200.

GO and KEGG pathway analyses

From intersection between the 4266 co-expressed genes and the 828 DEGs, we obtained a total of 183 genes, which were selected for GO and KEGG pathway analyses (Supplementary Fig. S1C). The most enriched GO and KEGG results are presented in Supplementary Fig. S1D-1G. Results of GO functional annotation indicated that the main biological processes of the 183 overlapping genes were 'metabolic process' (biological process category), 'nucleus' (cellular component category), and 'protein binding' (molecular function category). The results of KEGG pathway analysis indicated that Ambra1 was associated with cancer-related pathways, such as 'MAPK signaling pathway', 'chronic myeloid leukemia', 'VEGF signaling pathway', and 'pathways in cancer'.

Table 2. Relationships between Ambra1 expression and clinicopathological parameters. Her+: Her2 over-expressing subtype; TNBC: triple negative breast cancer

Clinicopathological parameters	Group	Number of patients	Ambra1 expression (mean±SD)	P-value
Age	≤60 years	601	11.5±0.59	0.023
	>60 years	489	11.42±0.61	
Gender	Female	1078	11.46±0.60	0.689
	Male	12	11.40±0.72	
ER	+	804	11.51±0.58	<0.0001
	-	236	11.31±0.65	
PR	+	695	11.51±0.58	0.001
	-	342	11.31±0.64	
Her2	+	1164	11.40±0.50	0.073
	-	560	11.50±0.61	
Stage	I–II	801	11.45±0.60	0.605
	III–IV	268	11.48±0.59	
T	T1–T2	908	11.46±0.59	0.979
	T3–T4	179	11.46±0.60	
N	N0	514	11.43±0.61	0.14
	N1–N3	576	11.49±0.56	
M	M0	907	11.48±0.57	0.004
	M1–M3	183	11.35±0.65	
	Luminal A	443	11.53±0.03	0.0082
Molecular subtype	Luminal B	126	11.43±0.05	
	Her2+	37	11.29±0.07	
	TNBC	115	11.35±0.07	
	Unclassified	370	11.44±0.03	

PPI network construction

To better understand the underlying molecular mechanism of the 183 overlapping genes, we established a PPI network using the online tool STRING. With a confidence score greater than 0.4, the PPI network was constructed with 182 nodes and 126 edges (Supplementary Fig. S1H).

Gene Set Enrichment Analysis (GSEA)

A total of 200 genes similar to Ambra1 were downloaded from the GEPIA database. The intersection between the 4266 co-expressed genes, the 828 DEGs, and the 200 similar genes was gathered for GSEA analysis (Fig. S6A). Twelve overlapping genes (ATG13, PRRC2B, C11orf49, MAPKBP1, CTDSP2, RERE, ZNF629, TMEM127, ZBTB4, TRIM44, RIC8A, and EFCAB14) were uploaded to the GSEA website and the expression levels of the 12 genes in various tissues and cell lines are presented in Fig. S6B -6C.

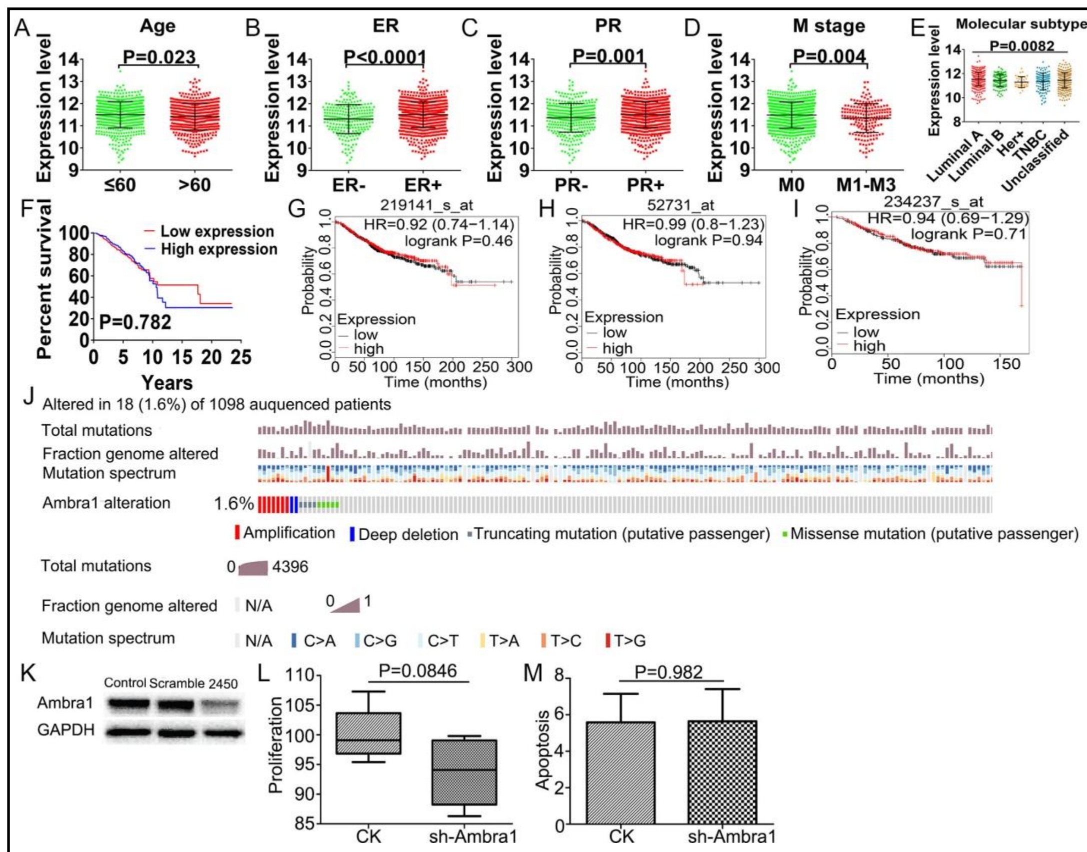

Fig. 6. Clinical significance and biological function of Ambra1 in BC. (A) Relationship between Ambra1 expression and age. (B) Relationship between Ambra1 expression and ER status. (C) Relationship between Ambra1 expression and PR status. (D) Relationship between Ambra1 expression and M stage. (E) Relationship between Ambra1 expression and molecular subtype. (F) Prognostic value of Ambra1 in BC based on data from TCGA (http://cancergenome.nih.gov/). (G) Prognostic value of Ambra1 in BC based on probe 219141_s_at from Kaplan–Meier plot (http://kmplot.com/analysis). (H) Prognostic value of Ambra1 in BC based on probe 52731_at of from Kaplan–Meier plot. (I) Prognostic value of Ambra1 in BC based on probe 234237_at from Kaplan-Meier plot. (J) Ambra1 alteration in BC based on data from cBioPortal (http://www.cbioportal.org/). (K) Western blotting examining the knock-down efficiency of Ambra1 in MDA-MB-231 cells. (L) Effects of Ambra1 on MDA-MB-231 cell proliferation. (M) Effects of Ambra1 on MDA-MB-231 cell apoptosis.

Discussion

In this study, we comprehensively investigated the contribution of Ambra1 to BC based on high-throughput data, including GEO, ArrayExpress, Oncomine, and TCGA. Finally, 25 microarray and RNA-seq datasets with 2460 BC samples were included. Ambra1 was found to be markedly up-regulated in BC tissues; furthermore, the Ambra1 level was significantly related to BC progression, especially metastatic status. Subsequently, we performed a microarray after Ambra1 knock-down *in vitro* and gathered co-expressed genes of Ambra1 from MEM. Eventually, the overlapping 183 genes were found to be enriched in several well-known cancer-related pathways, which could partially explain the effect of Ambra1 on cell proliferation inhibition in BC cells.

Ambra1 is an autophagy-related gene that can regulate Beclin1. Interplay between Ambra1 and Beclin1 has been reported previously. Here, we found their close correlation in BC based on data from TCGA (Fig. 7A), suggesting that Ambra1 may interact with Beclin1

to regulate autophagy and further affect the development of BC. Recent studies have confirmed the oncogenic role of Ambra1, an autophagy-related protein, in several types of cancers, but not in BC. The expression of Ambra1 protein in 73 cases of PDAC tissues was assessed using immunohistochemical staining based on a tissue-array. Among these 73 PDAC patients, Ambra1 protein expression rate was 63.9% (46/72), which was higher than in non-cancerous controls [10]. Immunohistochemistry was also performed to determine the Ambra1 expression in 65 hilar and extrahepatic cholangiocarcinomas. The expression pattern of Ambra1 protein was faint or weak in normal bile duct epithelium. By contrast, in cholangiocarcinoma tissues, strong expression of Ambra1 was observed in 71% cases (46/65) [11]. Ambra1 protein expression was investigated by both immunohistochemistry and Western blotting in tissue samples from both benign and malignant prostatic lesions. This pilot study revealed an increase in Ambra1 protein expression [12]. A similar expression pattern of Ambra1 was also reported in GC. Quantum dots immunofluorescence histochemistry was used to detect Ambra1 protein expression in GC tissue microarrays with 163 cancer samples and 20 gastric control tissues. Concurrently, Western blotting was also performed in the 10 paired fresh GC tissues with their controls. Consistent with findings in PDAC, cholangiocarcinoma, and prostate cancer, Ambra1 protein expression was clearly up-regulated in GC compared with non-cancerous gastric controls [14]. To the best of our knowledge, the expression level of Ambra1 has not previously been reported. The relationship between Ambra1 and BC was mentioned in only one previous study. Shen et al [34]. reported that docetaxel and cisplatin could induce autophagy in BC cells. Real-time polymerase chain reaction revealed that cisplatin could upregulate multiple autophagy-related genes, including Ambra1. However, the authors did not focus on the clinical and functional significance of Ambra1 in BC. In the present study, we collected and mined the publicly available high-throughput data to elucidate the potential role of Ambra1 in the tumorigenesis of BC. It is interesting that from 25 microarray and RNA-seq datasets involving 2460 BC samples, the constant up-regulation of Ambra1 could be observed by both SMD and SROC methods, though the individual studies showed some contradictory results. Additionally, the high expression of Ambra1 protein was corroborated by The Human Protein Atlas. Furthermore, data from cBioPortal also suggested that some genetic alterations of Ambra1 could occur in BC, including amplification and deep deletion. These results, for the first time, have shed light on the potential role of Ambra1 in BC.

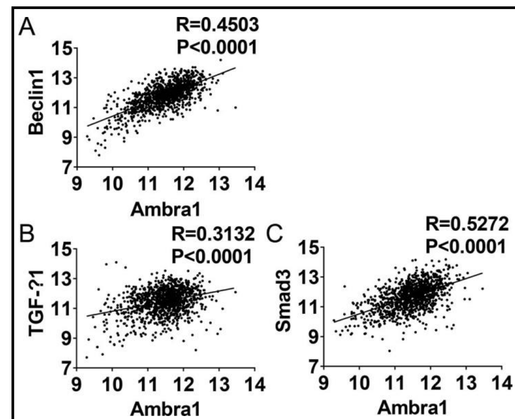

Fig. 7. Correlations between Ambra1 and Beclin1 as well as EMT-related genes based on data from TCGA. (A) Correlation between Ambra1 and Beclin1. (B) Correlation between Ambra1 and TGF-β1. (C) Correlation between Ambra1 and Smad3.

Ambra1 can induce the occurrence of tumors, and the driving role of Ambra1 in the progression of cancers has also been documented. Ambra1 could accumulate in prostate cancer and this high expression of Ambra1 was positively correlated with the Gleason score [12]. The expression of Ambra1 was positively associated with the epithelial–mesenchymal transition (EMT) in cholangiocarcinoma, which could partially explain that up-regulation of Ambra1 was closely associated with lymph node metastasis and poor survival [11]. Based on the findings from this study of cholangiocarcinoma, we examined the relationships between Ambra1 and two well-known EMT markers, TGF-β1 and Smad3. Positive correlations were noted between Ambra1 and these two EMT markers (Fig. 7B-7C) and the molecular mechanism underlying the influence of Ambra1 on EMT is worthy of in-depth investigation in the future. Moreover, in GC, Ambra1 played a role in accelerating tumor progression,

as over-expression of Ambra1 was clearly correlated with the depth of tumor invasion and lymph nodes metastasis. More importantly, over-expression of Ambra1 could be an independent indicator of overall survival of GC patients [14]. Furthermore, the earlier study on primary melanomas revealed a decline or even complete absence of Ambra1 expression in the epidermis overlying many stage I melanomas, which showed no relationship with the degree of melanoma epidermal invasion, and this lower or absent Ambra1 expression was not observed in benign nevi. These facts indicated that Ambra1 expression in the melanoma microenvironment may have prognostic potential. Essentially, this lower or absent Ambra1 expression was predominantly related to worse disease-free survival, with stratification for American Joint Committee on Cancer (AJCC) stage I disease. Thus, epidermal Ambra1 expression could act as a marker of disease progression for primary melanomas [13]. In the present study, we also observed that Ambra1 was greatly over-expressed in young patients with ER- and PR-positive BC. After classifying BC patients into different subtypes by the expression status of ER, PR, and Her2, we found that the expression level of Ambra1 was highest in the luminal A subtype and lowest in the Her2 over-expressing subtype. However, the precise mechanism needs to be further investigated. Surprisingly, Ambra1 expression was significantly higher in M0 stage than in M1–3 stage BC, which was inconsistent with findings in cholangiocarcinoma and GC. The biological function of Ambra1 may be tumor-specific. We also attempted to assess the prognostic role of Ambra1 in BC, but no significant results were obtained. Larger cohorts are required to further validate the current findings.

From the clinical observation above, we are interested in the mechanism of action of Ambra1 in the tumorigenesis and progression of BC. We therefore performed in-house microarray after Ambra1 knock-down in BC cells *in vitro*, which led to a slight suppression of cell proliferation. Altogether, 828 DEGs were obtained. To enhance the specificity of Ambra1-related genes, we gathered another group of 4266 co-expressed genes. Finally, the overlapping 183 genes were particularly enriched in several cancer-related pathways, including the MAPK signaling pathway, chronic myeloid leukemia, the VEGF signaling pathway, and pathways in cancer. Ambra1 may influence these well-known pathways and thereby affect the incidence and progression of BC. However, the molecular mechanism underlying the influence of Ambra1 in BC requires further *in vitro* and *in vivo* investigation.

Autophagy is the principal lysosomal-mediated process for the degradation/recycling of cellular debris and is a topic of intense debate in cancer medicine. In this study, we applied multiple detection methods to confirm that Ambra1, a vital autophagy-related gene, is up-regulated in BC tissues. The driving role of Ambra1 in the incidence and development of BC could be implemented via influencing several well-explored signaling pathways. However, the clinical significance of Ambra1 detection needs to be validated with more in-depth research.

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Disclosure Statement

The authors declare no conflicts of interest.

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