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ARTICLE

Saccharomyces cerevisiae-like 1 (SEC14L1) is a prognostic factor in breast cancer associated with lymphovascular invasion

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Abstract

Lymphovascular invasion is strongly related to breast cancer metastasis. However, the underlying mechanisms of lymphovascular invasion and its driver molecules in breast cancer remain to be defined. In this study, we explore differential expression of genes in large molecularly characterized and clinically annotated datasets of invasive breast cancer patients ($n = 8056$) coupled with histological review and strict definition for lymphovascular invasion status. The METABRIC series was used to identify genes associated with lymphovascular invasion, as defined using hematoxylin and eosin staining supplemented by immunohistochemistry, at the genomic/transcriptomic levels. *Saccharomyces cerevisiae*-like 1 (*SEC14L1*) was identified as one of the most significant genes associated with lymphovascular invasion. The prognostic significance of *SEC14L1* gene copy number and mRNA expression was further investigated in the METABRIC series and externally validated using the Breast Cancer Gene-Expression Miner v4.0. Protein expression of *SEC14L1* was also assessed using immunohistochemistry in series of early stage breast cancer using tissue microarrays. *SEC14L1* gene copy number gain was significantly associated with high histological grade and poor outcome. *SEC14L1* mRNA expression showed positive association with higher grade, lymph node metastasis, and poor outcome. SEC14L1 protein overexpression was significantly associated with lymphovascular invasion ($p < 0.0001$), higher grade ($p = 0.011$), HER2 positivity ($p = 0.036$), and shorter survival ($p = 0.00075$). Our findings specify *SEC14L1* as an independent prognostic factor in breast cancer. Its association, at both transcriptome and protein expression levels, with lymphovascular invasion and outcome could imply an important role in tumor progression. A further mechanistic insight into its molecular roles including potential therapeutic utility is warranted.

Introduction

Although several biomarkers associated with breast cancer progression and response to therapy have been identified, the exact molecular signatures for invasion machineries that lead to metastatic disease remains to be defined. Deciphering the driver genes/proteins that dictate the biological metastatic behavior of breast cancer is essential to understanding cancer progressive mechanisms and opening novel avenues for therapeutic interventions. Lymphovascular invasion is an independent prognostic parameter of poor outcome in invasive breast cancer and is major prerequisite for the development of metastasis [1–3]. Comprehension of the molecular mechanisms underlying lymphovascular invasion in breast cancer and unveiling its key-players could lead to unique therapeutic targets and improve risk prediction [4–6]. However, the sophistication of the

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molecular mechanisms underlying lymphovascular invasion as part of the invasion-metastasis cascade with involvement of several genes, diverse signaling pathways and interactions of tumor microenvironment in addition to the subjectivity of lymphovascular invasion morphological assessment in clinical samples renders this task highly challenging [6].

Advancement in high-throughput molecular and bioinformatics techniques coupled with thorough pathological assessment could help decipher lymphovascular invasion molecular regulators in breast cancer. Therefore, we have interrogated transcriptomic profiles of the large-scale cohort of invasive breast cancer patients of the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) following morphological and molecular assessment for lymphovascular invasion status to identify lymphovascular invasion differentially expressed genes [7]. Cytosolic factor *Saccharomyces cerevisiae*-like 1 (*SEC14L1*) was identified as one of the most significant genes associated with lymphovascular invasion using genomic data.

The aim of the present study was to evaluate the clinicopathological and prognostic significance of *SEC14L1* copy number alteration and mRNA expression in the large annotated cohort of the METABRIC. Associations of SEC14L1 protein expression with clinicopathological prognostic variables including lymphovascular invasion, intrinsic molecular subtypes, and patient outcome were assessed. Our interpretation of the molecular and prognostic impact of *SEC14L1* expression may unveil new insights on the complex pathways of lymphovascular invasion in breast cancer.

Material and methods

Analysis of differential gene expression

The METABRIC cohort [8, 9] is a large genomic and transcriptomic dataset from 1980 primary operable invasive breast cancer female patients. In this cohort, the estrogen receptor-positive and/or lymph node metastasis-negative patients were treated without adjuvant chemotherapy. The estrogen receptor-negative or lymph node-positive patients had adjuvant chemotherapy. No HER2-positive breast cancer patients had trastuzumab therapy. The extracted and purified DNA probes were hybridized to Affymetrix SNP 6.0 arrays (Affymetrix, Santa Clara, CA) at AROS Applied Biotechnology (Aarhus, Denmark). For RNA analysis, the Illumina Totalprep RNA amplification kit and Illumina Human HT-12 v3 Expression BeadChips (Ambion, Warrington, UK) were used as described previously [8].

We interrogated the Nottingham cases from the METABRIC cohort (discovery set) and gene expression levels were compared between lymphovascular invasion-positive and lymphovascular invasion-negative cases. The lymphovascular invasion status for all patients was evaluated morphologically using hematoxylin and eosin staining on full-face tumor sections. Besides, corresponding full-face tumor sections for each patient were stained immunohistochemically with CD34 and D2-40 (podoplanin, *PDPN*), [4] to refine the status of lymphovascular invasion. Cases were considered lymphovascular invasion negative by the absence of lymphovascular invasion in both hematoxylin and eosin-, and immunohistochemistry-stained section. Cases with discordant results or showing lymph node positive but lymphovascular invasion negative were excluded, to avoid bias caused by false-positive or false-negative diagnosis.

A linear model for microarray data and RNA-sequencing package (LIMMA) method [10] was applied for detecting differentially expressed genes/transcripts between lymphovascular invasion-positive and -negative groups. This supervised approach of differential gene expression analysis is compatible with the Affymetrix gene expression data. The Addenbrookes' Hospital cases, within the METABRIC cohort ($n = 521$), with defined lymphovascular invasion status were used to validate the results of the differential gene expression analyses (validation set). The top differentially expressed genes were ranked based on their p-value of association with lymphovascular invasion. Subsequently, the copy number alterations of the top differentially expressed genes were determined using the Affymetrix SNP6 Copy Number Inference Pipeline (Cancer Genomics Computation Analysis group of the Broad Institute, USA) [7]. Cytosolic factor *SEC14L1* was identified as one of the highly significant genes associated with lymphovascular invasion. The prognostic impact of *SEC14L1* mRNA expression was evaluated in the 1980 cases of METABRIC cohort and externally validated using the Breast Cancer Gene-Expression Miner v4.0 [$n = 5788$] [11].

Analysis of SEC14L1 protein expression

The Nottingham breast cancer series, which were included in the METABRIC cohort, were used in this study. All patients were treated without neoadjuvant treatment. Estrogen receptor, progesterone receptor, HER2, and Ki67 were stained and scored according to guideline recommendations and as previously published [12, 13]. The cutoff value of estrogen and progesterone receptors were determined as 1% [14]. Estrogen receptor-positive and HER2-negative tumors were considered as the luminal class with progesterone receptor-negative and high Ki67 (labelling index < 10%) cases were determined as luminal B subtype. Clinicopathological data and patients' outcome had been recorded and regularly updated. The recorded outcome data

Table 1 Association between *SEC14L1* copy number alterations and *SEC14L1* mRNA expression and clinicopathological parameters in the METABRIC cohort of invasive breast cancer ($n = 1980$)

Factors		Expression of *SEC14L1* (copy number alterations)					Expression of *SEC4L1* (mRNA)			
		Loss	Neutral	Gain	Total	*p*-Value	> Median	≤ Median	Total	*p*-Value
Tumor size	≥ 2 cm	30 (2.2%)	1114 (83.3%)	193 (14.4%)	1337	0.063	657 (49.1%)	681 (50.9%)	1338	0.10
	< 2 cm	13 (2.1%)	536 (87.3%)	65 (10.6%)	614		327 (53.1%)	289 (46.9%)	616	
Nodal status	Positive	20 (2.1%)	787 (84.1%)	129 (13.8%)	936	0.76	413 (44.0%)	525 (56.0%)	938	<0.0001
	Negative	23 (2.2%)	880 (85.1%)	131 (12.7%)	1034		575 (55.6%)	460 (44.4%)	1035	
Histological grade	Grade 3	22 (2.3%)	764 (80.3%)	165 (17.4%)	951	< 0.0001	355 (37.3%)	597 (62.7%)	952	< 0.0001
	Grade 1, 2	21 (2.2%)	834 (88.9%)	83 (8.8%)	938		589 (62.7%)	351 (37.3%)	940	
Molecular subtypes	Luminal A	14 (2.0%)	651 (90.8%)	52 (7.3%)	717	< 0.0001	481 (67.0%)	237 (33.0%)	718	<0.0001
	Luminal B	15 (3.1%)	358 (73.4%)	115 (23.6%)	488		252 (51.6%)	236 (48.4%)	488	
	HER2 enriched	5 (2.1%)	201 (84.1%)	33 (13.8%)	239		78 (32.5%)	162 (67.5%)	240	
	Basal-like	5 (1.5%)	275 (83.8%)	48 (14.6%)	328		74 (22.5%)	255 (77.5%)	329	
	Normal-like	4 (2.0%)	183 (92.0%)	12 (6.0%)	199		101 (50.8%)	98 (49.2%)	199	

comprised survival status, mean survival in months, recurrence of disease (including distant metastases), and cause of death. Breast cancer-specific survival was determined as the time from the day of surgery until the time of death from or with breast cancer.

Antigen-binding specificity for rabbit polyclonal anti-SEC14L1 antibody (HPA028703, Sigma Aldrich, UK) was examined by western blotting (WB) at 1:500. A set of different cell lysates was utilized: MCF7, HeLa, MDA-MB-231, Jurkat, human embryonic kidney 293, MDA-MB-468 (all obtained from the American Type Culture Collection, Rockville, MD, USA).

Before the immunohistochemistry assessment of SEC14L1 expression on tissue microarray, a subset of full-face tissue sections of breast cancer ($n = 20$) was immunohistochemically stained. Immunohistochemistry was performed on (4 µm) tissue microarray sections using Novocastra Novolink Polymer Detection Systems kit (Code: RE7280-K, Leica Biosystems, UK) following the manufacturer's protocol. Briefly, all full-face and tissue microarray sections were incubated on a hot plate at 60 °C for 10 min, then deparaffinized in xylene and rehydrated through descending grades of ethanol. SEC14L1 staining was performed utilizing anti-SEC14L1 primary antibody at 1:25 dilution in Leica Antibody Diluent, for 60 min. Finally, 3-3' Diaminobenzidine tetrahydrochloride (Novolink DAB substrate buffer plus) was freshly prepared and used as a chromogen. Counterstaining was performed using Meyer's hematoxylin for 6 min. Positive (anti-human-β-2-microglobulin; A0072, Dako) and negative controls were included in the staining runs. The cytoplasmic expression of SEC14L1 was assessed using the percentage of positivity (0–100%) and staining intensity (Negative: Score 0, Weak: Score 1, Moderate: Score 2, and Strong: Score 3). H-score of SEC14L1 was calculated as previous publication [15].

This work was approved by Nottingham Research Ethics Committee 2 under the title: Development of molecular genetic classification of breast cancer. All tissue samples included in this study were from patients who were consented before inclusion in the study cohort.

Statistical analysis

Statistical analysis was performed using SPSS (IBM SPSS Statistics, Version 22). The relationship between *SEC14L1* copy number alteration and mRNA expression was calculated using analysis of variance test with Bonferroni correction. Differences between two groups were assessed using Mann–Whitney test (non-normal distribution) to determine the associations between *SEC14L1* mRNA expression and lymphovascular invasion status. χ^2-test was used to evaluate the relationship between SEC14L1 expression and categorical variables. For dichotomization of the data, X-Tile (X-Tile Bioinformatics Software, Yale University, version 3.6.1) was used. Survival curves were generated by Kaplan–Meier survival analysis with differences in outcome assessed by Log Rank test. Cox's proportional hazard method was performed for multivariate analysis to identify the independent prognostic/predictive factors. The p-value ≤ 0.05 was considered significant.

Results

There was a strong positive correlation between *SEC14L1* mRNA expression and *SEC14L1* gene copy number gain; higher levels of *SEC14L1* mRNA were detected in cases with copy number gain compared with those with neutral copy number ($p < 0.0001$). *SEC14L1* mRNA expression

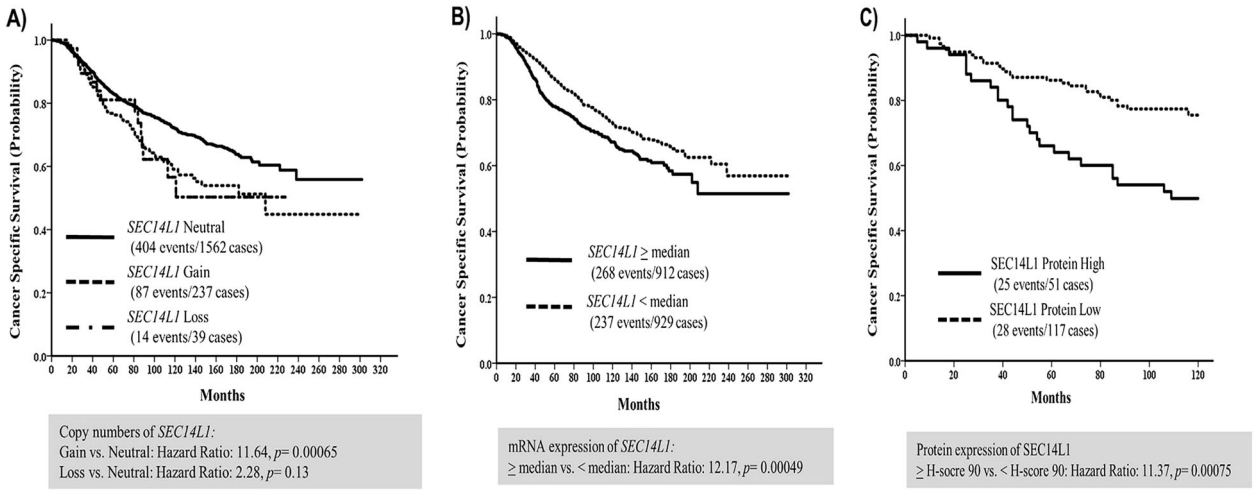

Fig. 1 Kaplan–Meier survival plots showing the association between *SEC14L1* copy numbers (**a**), mRNA expression (**b**), and SEC14L1 protein expression (**c**) and outcome

was significantly lower in the copy number loss group ($p < 0.0001$). Further analysis of *SEC14L1* copy number alteration revealed a significant association with histological grade 3 ($p < 0.0001$) and luminal B molecular subtype ($p < 0.0001$) (Table 1). The cutoff value of *SEC14L1* mRNA expression was determined at median value. The overexpression of *SEC14L1* mRNA was associated with the higher histological grade ($p < 0.0001$), axillary node metastasis ($p < 0.0001$), and the intrinsic molecular subtypes ($p < 0.0001$; Table 1).

In the METABRIC cohort, the survival of patients with *SEC14L1* copy number gain was significantly shorter than those of copy number neutral group ($p = 0.0007$). However, no difference of survival was observed between copy number alteration loss and neutral groups ($p = 0.13$) (Fig. 1a). High expression of *SEC14L1* mRNA conferred a significantly worse prognosis compared with low *SEC14L1* mRNA expression ($p = 0.00049$; Fig. 1b). External validation of the prognostic power of *SEC14L1* mRNA expression in the Breast Cancer Gene-Expression Miner v4.0 revealed that high *SEC14L1* mRNA expression was associated with poor prognosis ($p < 0.05$).In the METABRIC cohort, the survival of patients with *SEC14L1* copy number gain was significantly shorter than those of copy number neutral group ($p = 0.0007$). However, no difference of survival was observed between copy number alteration loss and neutral groups ($p = 0.13$) (Fig. 1a). High expression of *SEC14L1* mRNA is relevant with a worse prognosis compared with low *SEC14L1* mRNA expression ($p = 0.00049$; Fig. 1b). External validation of the prognostic power of *SEC14L1* mRNA expression in the Breast Cancer Gene-Expression Miner v4.0 revealed that high *SEC14L1* mRNA expression was associated with adverse prognosis ($p < 0.05$).

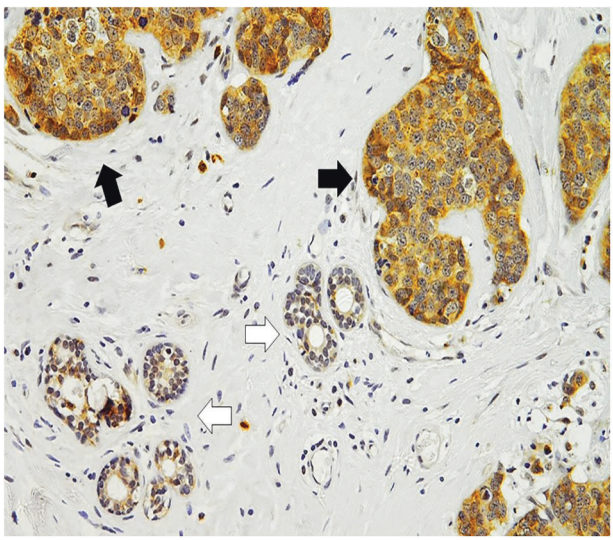

Fig. 2 SEC14L1 protein expression in normal and breast cancer tissue. Cytoplasmic SEC14L1 expression was overexpressed in breast cancer cells compared to the expression in epithelial cells of normal duct lobular units (black arrow: invasive carcinoma; white arrow: normal mammary gland)

SEC14L1 protein expression

Evaluation of WB supported a specific binding affinity of the anti-SEC14L1 primary antibody to single protein band for each cell lysate around the predicted molecular mass (77 kDa) of SEC14L1 protein, confirming the specificity of the antibody (Supplementary Figure 1). On full-face tissue sections, strong protein expression of SEC14L1 was detected in the cytoplasm of breast cancer cells (Fig. 2). On the other hand, weaker protein expression was observed in the adjacent normal epithelial tissue. Assessment of full-face sections revealed a homogeneous expression pattern for SEC14L1 protein

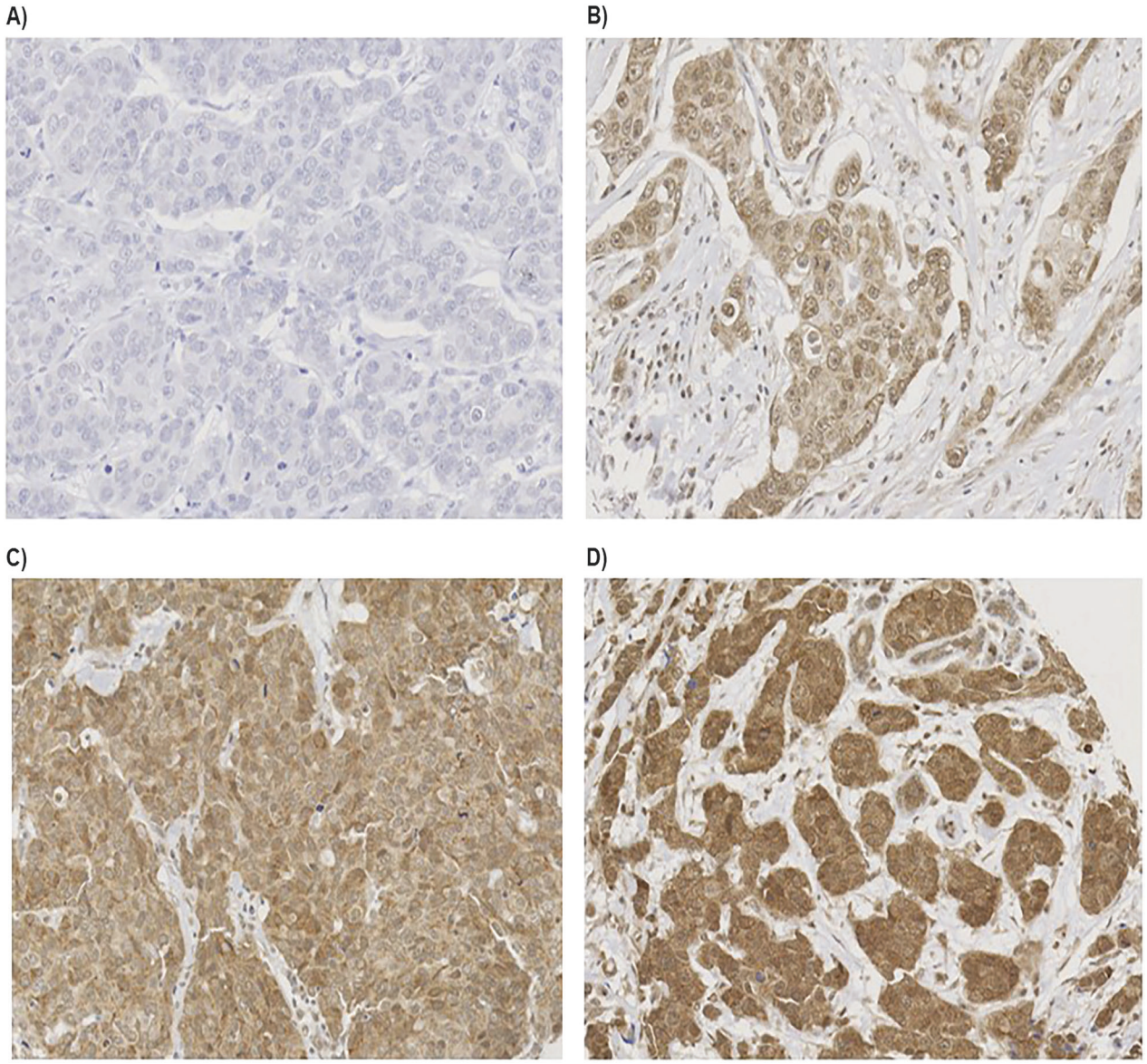

Fig. 3 Immunohistochemistry expression of SEC14L1 in tissue microarray images. SEC14L1 expression of the cytoplasm in cancer cells was distributed as follows; **a** no staining, **b** weak staining, **c** moderate staining, and **d** strong staining

confirming the reliability of tissue microarrays to detect its expression (Fig. 3). The optimal cutoff value of SEC14L1 protein expression was set at H score of 80. At this cutoff, 70% cases showed low/negative cytoplasmic expression, whereas positive/high cytoplasmic expression was detected in 30%.

There was an association between SEC14L1 protein expression and lymphovascular invasion status ($p < 0.0001$) and other variables of poor prognosis (Table 2). SEC14L1 expression was associated with a higher histological grade ($p = 0.011$), HER2 positivity ($p = 0.004$), and luminal B subtype ($p = 0.006$). Outcome analysis revealed a positive association between SEC14L1 protein expression and shorter survival ($p = 0.0008$; Fig. 1c). In univariate analysis, SEC14L1 protein expression ($p = 0.001$), histological grade ($p < 0.0001$), lymphovascular invasion status ($p = 0.002$), tumor size ($p = 0.005$), and nodal status ($p = 0.013$) were all significant predictors of outcome. Using multivariate analysis including prognostic variables significant in univariate analysis, SEC14L1 protein expression was an independent prognostic variable of survival ($p = 0.019$; Table 3).

Discussion

In the present research, we have tried to decipher the molecular mechanism underlying lymphovascular invasion in breast cancer. High-throughput molecular techniques

Table 2 Association between SEC14L1 protein expression and the clinicopathological factors

Factors		Expression of SEC14L1			
		Low	High	Total	*p*-Value
Tumor size	≥ 2 cm	68 (68.7%)	31 (31.3%)	99	0.75
	< 2 cm	49 (71.0%)	20 (29.0%)	69	
Nodal status	Positive	55 (67.1%)	27 (32.9%)	82	0.48
	Negative	62 (72.1%)	24 (27.9%)	86	
Histological grade	Grade 3	53 (60.9%)	34 (39.1%)	87	0.011
	Grade 1, 2	64 (79.0%)	17 (21.0%)	81	
Lymphovascular invasion	Positive	43 (51.8%)	40 (48.2%)	83	< 0.0001
	Negative	74 (88.1%)	10 (11.9%)	84	
Estrogen receptor	Positive	85 (69.1%)	38 (30.9%)	123	0.80
	Negative	32 (71.1%)	13 (28.9%)	45	
Progesterone receptor	Positive	66 (70.2%)	28 (29.8%)	94	0.96
	Negative	44 (69.8%)	19 (30.2%)	63	
HER2	Positive	14 (53.8%)	12 (46.2%)	26	0.036
	Negative	98 (74.2%)	34 (25.8%)	132	
Molecular subtypes	Luminal A-like	30 (88.2%)	4 (11.8%)	34	0.006
	Luminal B-like	39 (65.0%)	21 (35.0%)	60	
	HER2-positive	14 (53.8%)	12 (46.2%)	26	
	Triple negative	23 (85.2%)	4 (14.8%)	27	

Abbreviations: *ER* Estrogen receptor; *PgR* Progesterone receptor

coupled with bioinformatics and strict definition of lymphovascular invasion status were utilized to identify genes associated with lymphovascular invasion that can potentially be used as therapeutic targets. Following internal and external validation, *SEC14L1* was identified as one of the top differentially expressed genes associated with lymphovascular invasion and also with prognosis. Further examination of SEC14L1 at protein expression level confirmed its association with lymphovascular invasion and indicated its independent prognostic value in breast cancer.

SEC14L1 gene is located within a discrete region of 17q25 that frequently shows copy number alterations in breast cancer [16]. A proximate locus, the17q23 locus is well documented in breast cancer, and the key feature of this amplified locus is its oncogenic activity in breast cancer both in vitro and in vivo [17]. In this study, there was an association not only between *SEC14L1* copy number alteration and mRNA expression but also between copy number alteration and mRNA expression and lymphovascular invasion status. In a recent investigation of prostatic carcinoma, a prognostic 12-gene signature, including *SEC14L1*, associated with criteria of aggressive clinical outcome, which was also confirmed by real-time quantitative and immunohistochemistry assays [18]. SEC14L1 cytoplasmic protein overexpression was frequently found in the Transmembrane Protease, Serine 2 (*TMPRSS2*)/ Ets-related gene (*ERG*) fusion-positive prostate cancer, and the clinical and prognostic power of SEC14L1 strongly depends on this fusion status in prostate cancer [19]. SEC14-like proteins have been implicated in hepatocellular carcinomas [20].

TMPRSS2/*ERG* fusion has been revealed to be associated with several molecular alterations including deletion of the phosphatase and tensin homolog gene (*PTEN*) [21]. Several previous studies suggested that *PTEN* loss is strongly correlated with transcriptional instability and was associated with poor outcome of HER2-positive breast cancer [22, 23]. *TMPRSS2*/*ERG* fusion was also thought to be correlated with androgen receptor activity [24], transcriptional stability [25], and stem cell maintenance [26] in cancer. These mechanisms have essential roles in cell proliferation, adhesion, invasion and migration. Thus, *SEC14L1* may play a pivotal role in the regulation of cell growth/tissue development and cell adhesion, underlying lymphovascular invasion and metastasis in breast cancer.

Moreover, this study has revealed that SEC14L1 protein expression had a significant relationship with HER2 status and high histological grade. We previously reported that definite lymphovascular invasion was significantly correlated with HER2 positivity [1] and tumor microenvironment has a crucial role in the HER2 signaling pathway, invasion, and metastasis, including the development of lymphovascular invasion [27, 28]. A previous study suggested that SEC14L1 might have an important role in trafficking proteins on cell membrane [29]. SEC14L1 belongs to SEC14 cytosolic factor family that has a role in the intracellular transport system [30] and innate immunity [31]. SEC14L1 overexpression may be responsible for

Table 3 Univariate and multivariate survival analysis of clinicopathological factors and SEC14L1 protein expression in breast cancer

Factors		Univariate analysis			Multivariate analysis		
		Hazard ratio	95% CI	*p*-Value	Hazard ratio	95% CI	*p*-Value
SEC14L1	Low	Reference			Reference		
	High	2.5	1.43–4.21	0.001	2.0	1.12–3.54	0.019
Lymphovascular invasion	Negative	Reference			Reference		
	Positive	2.5	1.41–4.47	0.002	1.5	0.79–2.74	0.22
Tumor size	< 2 cm	Reference			Reference		
	≥ 2 cm	2.4	1.30–4.43	0.005	1.8	0.96–3.47	0.067
Nodal status	Negative	Reference			Reference		
	Positive	2.0	1.16–3.53	0.013	1.4	0.76–2.47	0.29
Histological grade	Grade 1–2	Reference			Reference		
	Grade 3	3.7	1.96–6.87	< 0.0001	2.7	1.41–5.15	0.003

enhanced intra-tumor signaling pathways including HER2 and influence the tumor microenvironment to promote tumor growth, promoting lymphovascular invasion and metastasis in breast cancer. Although SEC14L1 was associated with higher histological grade, the association with outcome was independent of grade. In this study, SEC14L1 was associated with lymph node metastasis at mRNA levels, and its expression at mRNA and protein levels was associated with outcome independent of other prognostic variables.

In conclusion, this study revealed and confirmed that SEC14L1 expression is a significant prognostic factor for breast cancer. Overexpression of SEC14L1 may have an important role in the development of lymphovascular invasion, breast cancer progression, and metastasis. Further functional assessment of SEC14L1 to determine its mechanistic roles and therapeutic potential in breast cancer is warranted.

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Author Contributions: REA, GAR, and EIO conceived of the study, contributed to study design, and provided samples and data. SSN, CC, MS, AMA, MA, TMS, and GAR collected data. D-RM and NCC provided technical support. SSN carried out experiments. SSN, AMA, JC, and KS analysed and interpreted data, and generated the figures and tables. All authors contributed to drafting and reviewing the manuscript, and approved the submitted and final version.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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