Squalene epoxidase expression is associated with breast tumor progression and with a poor prognosis in breast cancer

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Abstract. Differentially expressed genes (DEGs) have been previously identified using massive parallel RNA sequencing in matched normal, breast cancer (BC) and nodal metastatic tissues. Squalene epoxidase (SQLE), one of these DEGs, is a key enzyme in cholesterol synthesis. The aim of the present study was to investigate the potential involvement of SQLE in the tumorigenic process of BC and to determine its association with the clinical outcome of BC. SQLE mRNA expression was measured using reverse transcription-quantitative PCR in 10 pairs of ductal carcinoma *in situ* (DCIS) and BC tissues and their adjacent normal tissues. Immunohistochemical staining of SQLE on tissue microarray was performed in 26 normal breast, 79 DCIS and 198 BC samples. The role of SQLE as a prognostic biomarker in patients with BC has been verified using BreastMark. SQLE mRNA expression was significantly increased in DCIS and BC tissues compared with that in their adjacent normal tissues. High SQLE expression was detected in 0, 48.1 and 40.4% of normal breast, DCIS and BC tissues, respectively. SQLE expression in DCIS and BC tissues was significantly higher than that in normal breast tissues. High SQLE expression was observed in DCIS with higher nuclear grade, comedo-type necrosis and HER2 positivity. High SQLE expression in BC was associated with larger tumor size, nodal metastases, higher stage, HER2 subtype and distant metastatic relapse. High SQLE expression was associated with poor disease-free and overall survival, and independently predicted poor disease-free survival in patients with BC. Following BreastMark analysis, high SQLE mRNA expression in BC was significantly associated with a poor prognosis in the 'all', lymph node negative, lymph node positive, luminal A subtype and luminal B subtype groups. Therefore, SQLE expression may be upregulated during the tumorigenic process of BC,

and high SQLE expression may be a useful biomarker for predicting a poor prognosis in patients with BC.

Introduction

In Korean women, breast cancer (BC) is one of the most common type of cancer (1). Despite the significant improvements in the overall survival and quality of life for women with BC, BC remains a leading cause of cancer-associated deaths (2). Therefore, it is necessary to develop new prognostic and therapeutic markers for patients with BC.

Cancer cells frequently exhibit altered cellular metabolism, which can mediate tumor progression and can be used for therapeutic purposes (3). Cholesterol is a unique lipid that is crucial for membrane formation, cell proliferation and cell differentiation (4). The critical role of cholesterol in the pathogenesis of various types of cancer, such as prostate and breast cancer, has been recognized in tumor cell proliferation, survival and treatment resistance (4-6). Moreover, several studies have revealed that inhibition of cholesterol synthesis at different steps results in human cancer cell death in both *in vitro* and *in vivo* models (3,4,7). Overall, dysregulation of cholesterol metabolism may be a promising new therapeutic target for cancer treatment.

Squalene epoxidase (SQLE) is one of the rate-limiting enzymes in cholesterol synthesis by catalyzing the first step of squalene oxygenation (8). Previous studies have revealed that dysregulation of SQLE expression is involved in the molecular pathogenesis of various types of cancer, such as prostate cancer (9,10), hepatocellular carcinoma (11), pancreatic cancer (12), esophageal squamous cell carcinoma (13) and squamous lung cancer (14). SQLE has been proposed as a new molecular marker to predict a poor prognosis in the aforementioned types of cancer (9-14).

Several studies have already been conducted with the aim of exploring the potential effect of the dysregulation of SQLE expression in BC (15-26). SQLE is involved in the maintenance of lipid droplet homeostasis in BC (15). Additionally, SQLE is involved in the process by which normal mammary fibroblasts induce a reversion of the malignant phenotype in primary BC (16). SQLE overexpression is more prevalent in groups with an unfavorable prognosis of stage I/II estrogen receptor-positive (ER+) BC (17), early-onset BC (18) and African-American patients with luminal A BC (19). Increased

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SQLE expression in patients with ER+ BC has been associated with poor response to endocrine therapy (20). Furthermore, high SQLE expression has been associated with increased risk of BC recurrence (21-23). SQLE copy number amplification has been associated with its overexpression and poor prognosis (24). MYC gene amplification and aberrant SQLE methylation have also been observed in aggressive BC (25). Brown *et al* (26) have confirmed that SQLE is a true oncogene by clinically relevant amplification in BC. Brown *et al* (26) have demonstrated that SQLE overexpression represents an independent adverse prognostic factor and that SQLE inhibition may be a novel therapeutic target for BC. Most of the aforementioned SQLE studies in BC have been based on a molecular approach. Although ductal carcinoma *in situ* (DCIS) is a non-invasive cancerous lesion of the breast (27), to the best of our knowledge, there are no studies on the profile of SQLE expression during BC progression, including DCIS.

We have previously performed massive parallel RNA sequencing (RNA-Seq) analysis in 21 samples (normal, cancer and nodal metastases) from 7 patients with ER+, HER2- BC, revealing SQLE as one of the differentially expressed genes (DEGs) (28). Therefore, the present study evaluated the potential involvement of SQLE in the tumorigenic process of BC. Additionally, whether SQLE detection by immunohistochemistry may predict the prognosis in patients with BC was investigated. SQLE expression was examined in 10 pairs of DCIS and BC tissues and their adjacent normal tissues at the mRNA level by reverse transcription-quantitative PCR (RT-qPCR). In addition, immunohistochemical staining of SQLE expression on tissue microarray (TMA) was performed in 26 normal breast, 79 DCIS and 198 BC samples. The role of SQLE as a prognostic biomarker in patients with BC was then verified using BreastMark (29).

Materials and methods

Validation of SQLE RNA-Seq data by RT-qPCR. We have previously generated comprehensive gene expression profiles of matched normal, cancer and lymph node metastatic tissues from 7 patients with ER+, HER2- BC using RNA-Seq analysis (28). To validate the RNA-Seq data, SQLE mRNA expression was analyzed by RT-qPCR, as previously described (30). The isolated RNA used for RNA-Seq in our previous study (28) was used for RT-qPCR. The qPCR reaction was performed in a 7500 Fast Real-Time PCR System using TaqMan® Gene Expression Master Mix (Thermo Fisher Scientific, Inc.) using the following thermocycling conditions: Initial denaturation for 30 sec at 95˚C, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 60 sec. The following probes (Thermo Fisher Scientific, Inc.) were used: Hs01123768_m1 (SQLE) and Hs02758991_g1 (GAPDH). The $2^{-\Delta\Delta Cq}$ method (31) was used for data analysis and the value of $2^{-\Delta\Delta Cq}$ indicated the fold change in SQLE expression normalized to GAPDH expression.

Samples for SQLE mRNA expression in DCIS and BC tissues and their adjacent normal tissues. New frozen samples of DCIS and BC tissues and their adjacent normal tissues (≥2 cm from DCIS/BC tissues) were provided by the Biobank of Chonnam National University Hwasun Hospital (Jeollanam,

Republic of Korea), which is a member of the Korea Biobank Network, and were used for RT-qPCR, as aforementioned. The present study included 10 female patients each with DCIS or BC. The mean age in patients with DCIS was 57.2 years (median age, 57.6 years; age range, 48-73 years), while the mean age in patients with BC was 56.6 years (median age, 53.0 years; age range, 39-86 years).

SQLE expression in normal, DCIS and BC tissues
Patients and tissues. Tissues were fixed in 10% formalin for 12-24 h at room temperature, cut into <3-mm-thick sections and embedded in paraffin. Formalin-fixed paraffin-embedded (FFPE) samples of normal breast tissues with no pathological lesions (n=26), DCIS (n=79) and BC (n=198) were selected from female patients within Chonnam National University Hospital (Gwangju, Republic of Korea) and Chonnam National University Hwasun Hospital (Jeollanam, Republic of Korea). The mean age in patients with no pathological lesions was 44.3 years (median age, 45.5 years; age range, 25-69 years), the mean age in patients with DCIS was 49.3 years (median age, 48.0 years; age range, 27-73 years) and the mean age in patients with BC was 46.6 years (median age, 45.0 years; age range, 26-89 years). Patients diagnosed with BC between January 1997 and December 2002 were included in the present study and had a follow-up of ≥10 years. After surgery, the patients underwent standard radiation therapy or adjuvant systemic therapy (hormone therapy or chemotherapy), according to the medical insurance program controlled by the Ministry of Health and Welfare of Korea. Medical records were reviewed to obtain clinicopathological information including patient outcome. ER, progesterone receptor (PR) and HER2 expression was assessed according to the American Society of Clinical Oncology/College of American Pathologists guidelines (32-34). DCIS samples between February 2005 and December 2011 were selected based on the availability of FFPE samples.

TMA construction. TMA blocks were constructed using one representative FFPE block in each case. Three cores of 1-mm diameter for BC and two cores of 2-mm diameter for normal breast and DCIS were punched from the donor block.

Immunohistochemistry and evaluation of immunostaining. SQLE expression was examined by immunohistochemical staining using the Bond-max automatic device (Leica Microsystems, Inc.), as previously described (35). Mouse polyclonal antibody against SQLE (1:200; cat. no. 042278; United States Biological) was used. According to a previous study (36), SQLE immunoreactivity was scored as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong), based on the intensity of cytoplasmic staining. SQLE expression was evaluated by light microscopy (magnification, x200). Samples with intensity staining scores of 0-2 were considered as low SQLE expression, while scores of 3 were defined as high SQLE expression.

Validation of SQLE as a prognostic biomarker using BreastMark. SQLE was further analyzed to validate its prognostic value in patients with BC using the BreastMark database, as previously described (29).

Statistical analysis. The data of SQLE expression in matched normal, cancer and lymph node metastatic tissues from seven
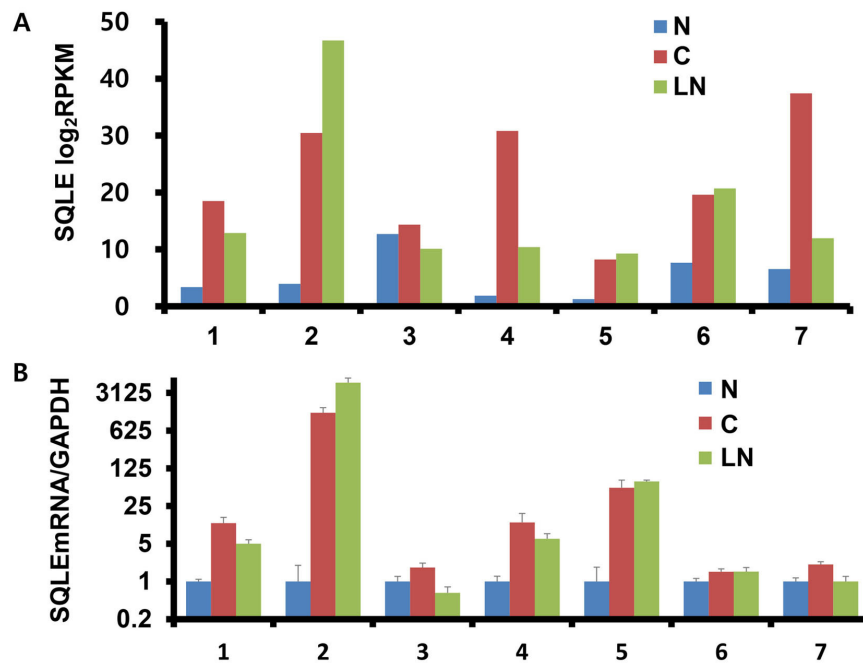
Figure 1. SQLE expression assessed via (A) RNA-Sequencing and (B) reverse transcription-quantitative PCR in matched N, C and LN tissues in seven patients with breast cancer. FPKM, fragments per kilobase of transcript per million mapped reads; N, normal; C, cancer; LN, lymph node metastatic; SQLE, squalene epoxidase.

patients were analyzed using one-way repeated measures ANOVA followed by post-hoc analysis with Fisher's Least Significant Difference adjustment for multiple comparisons. SQLE mRNA expression according to RT-qPCR in the DCIS and BC tissues and their adjacent normal tissues were compared using a paired Student's t-test (two-sided). Categorical nominal variables were tested using the χ^2 test or Fisher's exact test. Linear-by-linear association was added to test for the trend. Univariate survival analysis was performed according to the Kaplan-Meier method and the differences in survival curves were assessed with the log-rank test. Cox's proportional hazard model was used for multivariate analysis. SPSS (v25 for windows; IBM Corp.) was used for all statistical analyses. P<0.05 was considered to indicate a statistically significant difference.

Results

Validation of SQLE RNA-Seq data by RT-qPCR. SQLE expression was assessed by RNA-Seq (Fig. 1A) and RT-qPCR (Fig. 1B) in matched normal, cancer and nodal metastatic tissues in seven patients with BC. In the RNA-Seq results, SQLE expression (mean ± SD) was upregulated in BC tissues compared with in adjacent normal breast tissues (22.8±10.4 vs. 5.3±3.9 for BC vs. normal, respectively; fold change, 3.915; P=0.007) and subsequently unchanged when comparing nodal metastatic tissues with corresponding BC tissues (17.4±13.5 vs. 22.8±10.4 for nodal vs. BC; fold change, -1.3014; P=0.354) (data not shown). For RT-qPCR, the unamplified total RNA (from the same batch used for RNA-Seq) was used as the template. Although no significant differences among groups were observed, SQLE expression was mainly upregulated in BC and nodal metastatic tissues compared with in adjacent normal breast tissues (data not shown). Overall, the results obtained with the two different techniques were consistent for ~70% (5/7) of the samples tested.

SQLE mRNA in DCIS and BC tissues and their adjacent normal breast tissues. SQLE expression was assessed by RT-qPCR in 10 frozen DCIS tissues (Fig. 2A) and 10 BC tissues (Fig. 2B) and their respective adjacent normal breast tissues. The expression levels of SQLE mRNA (mean ± SD) were significantly increased in DCIS and BC tissues compared with in their adjacent normal breast tissues (6.27±7.54 vs. 1.00±0.00 for DCIS vs. normal, P<0.05; 14.02±22.95 vs. 1.00±0.00 for BC vs. normal, P<0.05) (data not shown). No significant differences in SQLE mRNA expression (mean ± SD) were observed between DCIS and BC (6.27±7.54 vs. 14.02±22.95, respectively; P=0.323) (data not shown).

SQLE expression in normal, DCIS and BC tissues. Normal breast tissues exhibited weak cytoplasmic SQLE expression (Fig. 3A and B). In DCIS and BC tissues, the carcinoma cells displayed variable SQLE expression (Fig. 3C-F). Table I summarizes SQLE expression in normal breast, DCIS and BC tissues. High SQLE expression was detected in 0/26 (0%) of normal breast tissues, 38/79 (48.1%) of DCIS samples, and 80/198 (40.4%) of BC samples. Differential SQLE expression was noted in the DCIS and BC groups compared with in the normal breast group (P<0.05, linear-by-linear association for trend). SQLE expression in DCIS and BC tissues was significantly higher compared with in normal breast tissues (DCIS vs. normal, 48.1 vs. 0%, P<0.001; BC vs. normal, 40.4 vs. 0%, P<0.001). There was no significant difference in the expression levels of SQLE between DCIS and BC (DCIS vs. BC, 48.1 vs. 40.4%, P=0.242).

Tables II and III summarize the associations between SQLE expression and the clinicopathological features of patients with DCIS and BC, respectively. High SQLE expression in DCIS tissues was associated with nuclear grade (P<0.01), comedo-type necrosis (P<0.05) and HER2
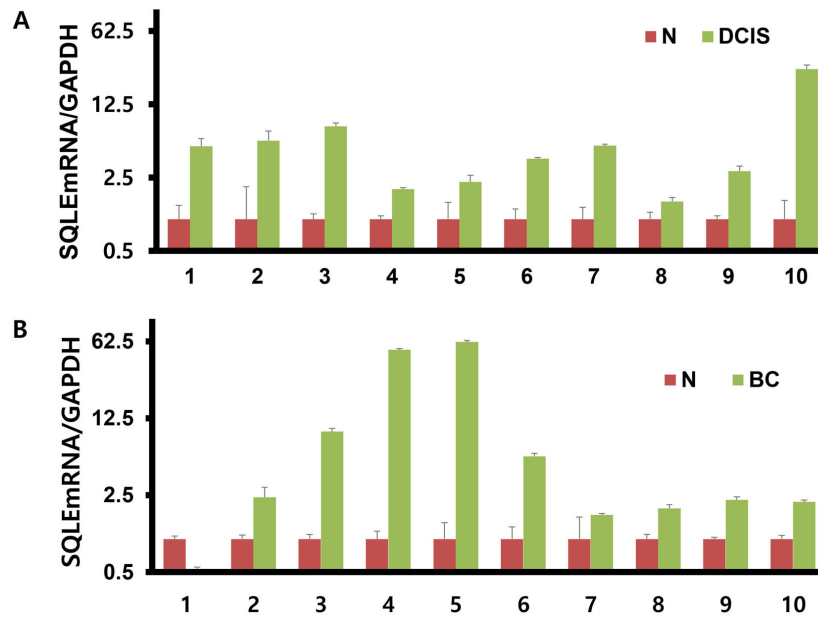
Figure 2. SQLE mRNA expression assessed via reverse transcription-quantitative PCR in (A) DCIS and (B) BC tissues and their adjacent N breast tissues. N, normal; DCIS, ductal carcinoma *in situ*; BC, breast cancer; SQLE, squalene epoxidase.
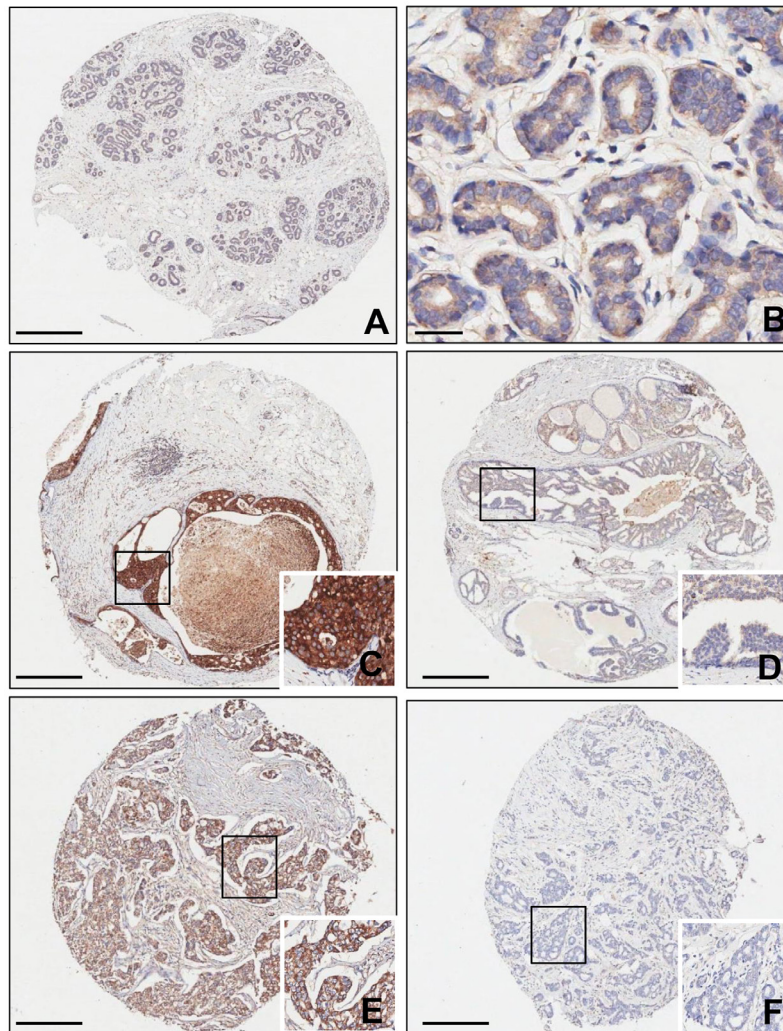

Figure 3. SQLE expression in normal breast, DCIS and BC tissues. (A and B) Normal breast tissue exhibited weak cytoplasmic SQLE expression (A: magnification, x4; scale bar, 500 μm; B: magnification, x400; scale bar, 60 μm). (C) High and (D) low SQLE expression in the cytoplasm of DCIS tissues. (E) High and (F) low SQLE expression in the cytoplasm of BC tissues. Magnification, x4; scale bar, 500 μm (inlet magnification, x400). DCIS, ductal carcinoma *in situ*; BC, breast cancer; SQLE, squalene epoxidase.
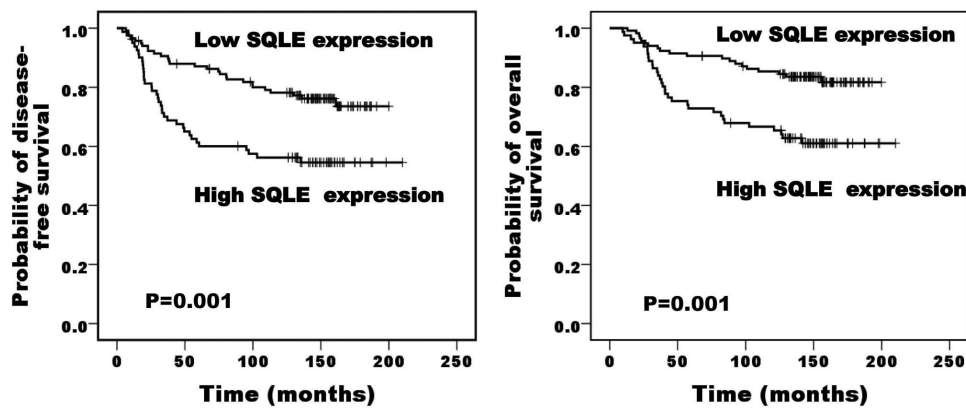
Figure 4. Survival of patients grouped according to SQLE expression. High SQLE expression predicted a significantly poor disease-free survival and overall survival (P=0.001 and P=0.001, respectively). SQLE, squalene epoxidase.

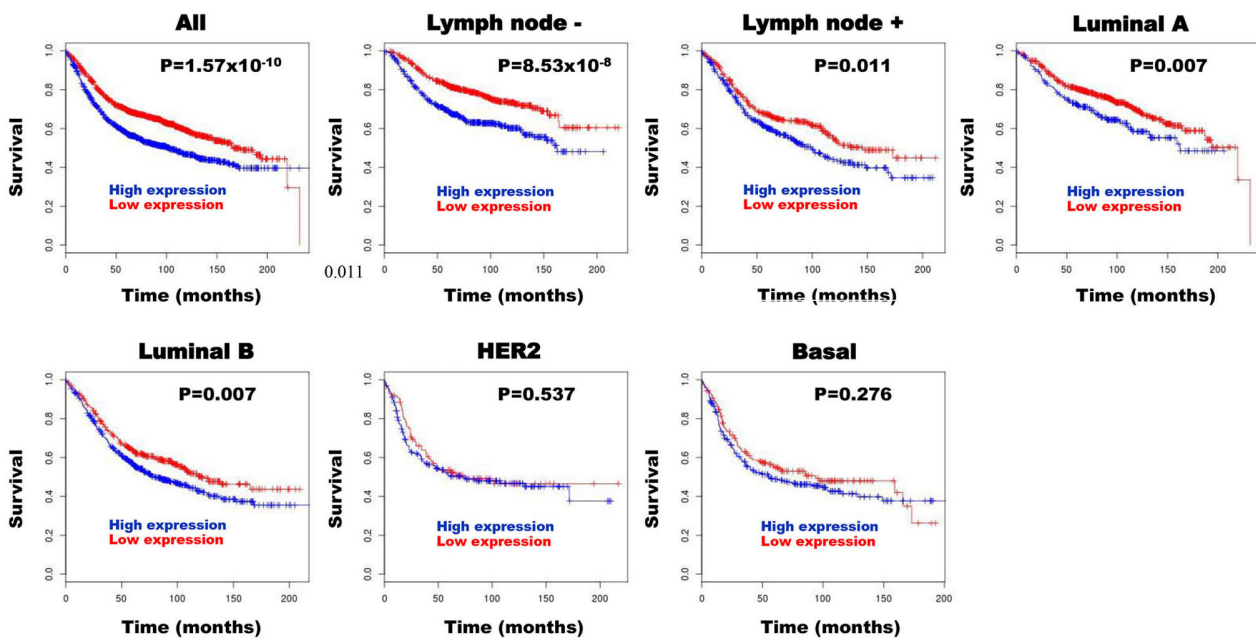Figure 5. Prognostic role of squalene epoxidase expression in breast cancer assessed using BreastMark.

status (P<0.01) (Table II). Although not statistically significant, the percentage of high SQLE expression was higher in patients with recurrence (5/7; 71.4%) than in patients without recurrence (33/72; 45.8%) (Table II). High SQLE expression in BC tissues was associated with tumor size (P<0.05), nodal metastases (P<0.001), stage (P<0.01), molecular subtype (P<0.05) and distant metastatic relapse (P<0.05) (Table III).

Summary of survival analysis in patients with BC. Table IV summarizes the results of univariate survival analysis according to log-rank test for Kaplan-Meier survival analysis. Patients with high SQLE expression exhibited a poor prognosis for disease-free survival and overall survival compared with those with low expression (P=0.001 and P=0.001, respectively; Fig. 4). Lymph node status and SQLE expression were independent poor prognostic factors for disease-free survival, while lymph node status was the only independent poor prognostic factor for overall survival (Table V).

Validation of SQLE as a prognostic biomarker using BreastMark. Following BreastMark analysis, high SQLE mRNA expression in patients with BC was significantly associated with poor prognosis in all groups [hazard ratio (HR), 1.467; P=1.57x10^{-10}; n=2,652), lymph node negative group (HR, 1.781; P=8.53x10^{-8}; n=1,183), lymph node positive group (HR, 1.337; P=0.011; n=744), luminal A subtype (HR, 1.427; P=0.007; n=823) and luminal B subtype (HR, 1.284; P=0.007; n=1,013) (Fig. 5). However, high SQLE mRNA expression was not associated with prognosis in patients with HER subtype (HR, 1.117; P=0.537; n=286) and basal subtype (HR, 1.169; P=0.276; n=424) (Fig. 5).

Discussion

SQLE expression is associated with pathogenesis and clinical outcome in various types of cancer (9-14). The present study demonstrated the upregulation of SQLE mRNA expression in DCIS and BC tissues compared with in their adjacent normal

Table I. SQLE expression in normal breast, ductal carcinoma *in situ* and breast cancer tissues.

Type of tissue	High SQLE expression, n/total n (%)	P-value
Normal	0/26 (0.0)	0.018[a]
Ductal carcinoma *in situ*	38/79 (48.1)	
Breast cancer	80/198 (40.4)	

[a]Linear-by-linear association. Normal vs. ductal carcinoma *in situ*, P<0.001; normal vs. breast cancer, P<0.001; ductal carcinoma *in situ* vs. breast cancer, P=0.242. SQLE, squalene epoxidase.

Table II. Association between SQLE expression and clinicopathological parameters of patients with ductal carcinoma *in situ* (n=79).

Characteristics	High SQLE expression, n/total n (%)	P-value[a]
Age, years		>0.05
≤50	21/45 (46.7)	
>50	17/34 (50.0)	
Size, cm		>0.05
≤2.5	21/45 (46.7)	
>2.5	17/34 (50.0)	
Nuclear grade		<0.01
1	1/7 (14.3)	
2	19/46 (41.3)	
3	18/26 (69.2)	
Comedo-type necrosis		<0.05
No	11/32 (34.4)	
Yes	27/47 (57.4)	
Estrogen receptor-α		>0.05
Negative	17/28 (60.7)	
Positive	21/51 (41.2)	
HER2		<0.01
Negative	18/51 (35.3)	
Positive	20/28 (71.4)	
Recurrence		>0.05[b]
No	33/72 (45.8)	
Yes	5/7 (71.4)	

[a]Analyzed by χ^2 test; [b]Analyzed by Fisher's exact test. SQLE, squalene epoxidase.

Table III. Association between SQLE expression and clinicopathological parameters of patients with breast cancer (n=198).

Characteristics	High SQLE expression, n/total n (%)	P-value[a]
Age, years		>0.05
≤46	40/112 (35.7)	
>46	41/86 (47.7)	
Histopathologic type		>0.05
Invasive ductal carcinoma, NOS	67/171 (39.2)	
Invasive lobular carcinoma	14/25 (56.0)	
Mucinous carcinoma	0/2 (0.0)	
Tumor size, cm		<0.05
≤2	11/35 (31.4)	
2-5	51/133(38.3)	
>5	19/30 (63.3)	
Number of nodal metastasis		<0.001
0	29/105 (27.6)	
1-3	25/51 (49.0)	
4-9	13/25 (52.0)	
≥10	14/17 (82.4)	
Histological grade		>0.05
1	4/26 (15.4)	
2	47/101 (46.5)	
3	30/71 (42.3)	
Stage		<0.01
I	11/35 (31.4)	
II	37/112 (33.0)	
III	33/51 (64.7)	
Estrogen receptor-α		>0.05
Negative	40/87 (46.0)	
Positive	41/111 (36.9)	
Progesterone receptor		>0.05
Negative	39/87 (44.8)	
Positive	42/111 (37.8)	
HER2		>0.05
Negative	64/162 (39.5)	
Positive	17/36 (47.2)	
Molecular subtypes		<0.05
Luminal	50/135 (37.0)	
HER2	14/21 (66.7)	
Triple negative	17/42 (40.5)	
Distant metastatic relapse		<0.05
No	52/146 (35.6)	
Yes	29/52 (55.8)	

[a]Analyzed by χ^2 test. NOS, not otherwise specified; SQLE, squalene epoxidase.

breast tissues. SQLE expression was associated with breast tumor progression and with a poor clinical outcome in patients with BC.

Cholesterol is the main sterol that is synthesized in all animal cells and is an essential structural component of cell membrane (4). An association between cholesterol and cancer has been previously recognized (6). Moreover, previous studies have indicated that inhibition at different stages of cholesterol synthesis contributes to inhibition of tumor cell proliferation, cell death and resistance to therapies in cancer (3,4,7). Overall,

Table IV. Univariate analysis of prognostic factors in patients with breast cancer.

Characteristics	Disease-free survival (P-value)	Overall survival (P-value)
Age (≤46 vs. >46 years)	0.972	0.522
Histological type (invasive carcinoma of no special type vs. other types)	0.076	0.409
Tumor size (pT1 vs. pT2 vs. pT3)	<0.001	<0.001
Lymph node status (pN0 vs. pN1 vs. pN2 vs. pN3)	<0.001	<0.001
Histological grade (1 vs. 2 vs. 3)	0.513	0.105
Stage (I vs. II vs. III vs. IV)	<0.001	<0.001
Hormonal therapy (no vs. yes)	0.395	0.295
Chemotherapy/radiotherapy (no vs. yes)	0.026	0.017
Estrogen receptor-α status (negative vs. positive)	0.430	0.424
Progesterone receptor status (negative vs. positive)	0.527	0.625
HER-2 status (negative vs. positive)	0.966	0.627
Squalene epoxidase expression (low vs. high)	0.001	0.001

Table V. Multivariate analysis with Cox's proportional hazard model for prognostic factors in patients with breast cancer.

Characteristics	Disease-free survival			Overall survival		
	HR	95% CI	P-value	HR	95% CI	P-value
Age (≤46 vs. >46 years)	0.072	0.566-1.539	0.788	0.038	0.597-1.877	0.845
Tumor size (≤5 vs. >5 cm)	2.885	0.291-1.092	0.089	1.492	0.320-1.303	0.222
Lymph node status (negative vs. positive)	6.443	0.212-0.819	0.011	4.922	0.170-0.896	0.027
Stage (I/II vs. III)	0.440	0.394-1.584	0.507	1.885	0.265-1.263	0.170
Chemotherapy/radiotherapy (no vs. yes)	1.285	0.193-1.551	0.257	1.484	0.096-1.730	0.223
Squalene epoxidase expression (low vs. high)	4.079	0.354-0.985	0.043	3.561	0.318-1.022	0.059

HR, hazard ratio.

dysregulation of cholesterol metabolism may be a new therapeutic target in cancer treatment.

SQLE encodes squalene epoxidase, one of the major enzymes in the late stages of cholesterol synthesis (8). SQLE catalyzes the oxidation of squalene to 2,3-oxidosqualene (squalene epoxide), and its inhibition can affect the synthesis of sterols and of the cell membrane, or even cell growth (3). Previous studies have indicated that dysregulation of SQLE is involved in the molecular pathogenesis of various types of cancer and has also been associated with poor prognosis (9-14). SQLE has been associated with radiation resistance in pancreatic cancer (12) and metastasis in esophageal squamous cell carcinoma (13), as well as with poor outcomes in patients with prostate cancer (9,10), hepatocellular carcinoma (11) and squamous lung cancer (14).

Several studies have already been conducted to explore the potential role of the dysregulation of SQLE in BC (15-26). Polycarpou-Schwarz et al (16) identified a new microprotein gene, Cancer-Associated Small Integral Membrane Open reading frame 1 (CASIMO1), utilizing a microarray approach. CASIMO1 was found to be overexpressed and important for proper proliferation of breast cancer cells, and exerted its effects through interactions with SQLE maintaining lipid

droplet homeostasis (16). Römer et al (15) assessed the ability of normal human mammary fibroblasts (HMFs) to induce reversion of the malignant phenotype of primary breast carcinoma cells in a three-dimensional cell culture model. The reversion of the malignant phenotype was detected in 5/13 primary breast carcinoma cell co-cultures with HMFs (15). Gene expression analysis revealed that SQLE expression was downregulated in the reverted cases compared with in the non-reverted cases (15). These findings were consistent with the role of SQLE in regulating BC progression and inhibition of differentiation (15). cDNA microarrays reported that SQLE was differentially expressed in BC and that SQLE expression was increased dramatically in cancer tissues compared with in normal and cancer-adjacent normal tissues (18,19). In the present study, SQLE expression was directly compared in sets of matched normal breast, BC and nodal metastatic tissues using RNA-Seq and RT-qPCR. In the RNA-Seq data, SQLE expression was upregulated in BC tissues compared with in matched normal breast tissues, and was unchanged when comparing nodal metastatic tissues with matched BC tissues. Based on changes in gene expression during progression from normal tissue to cancer tissue and then to metastatic node of BC, SQLE may belong to genes

associated with tumorigenesis (normal-cancer transition) rather than metastasis (cancer-metastasis transition).

Although BC is believed to develop from DCIS (27), to the best of our knowledge, there are no studies that have examined the association between SQLE and tumor progression in BC, including DCIS lesions. In the present study, mRNA expression levels of SQLE in frozen DCIS and BC tissues and their adjacent normal breast tissues were assessed using RT-qPCR. The present data revealed that SQLE mRNA expression was significantly increased in DCIS and BC tissues compared with in adjacent normal tissues. This result demonstrated the oncogenic properties of SQLE in BC as well as in DCIS. However, there were no significant differences in SQLE mRNA expression between DCIS and BC. In accordance with the RT-qPCR findings, immunohistochemical analysis revealed that SQLE expression in DCIS and BC was significantly higher than that in normal breast tissues, but there was no significant difference in SQLE expression between DCIS and BC tissues. These results suggest that SQLE may serve an essential role in BC progression and may be especially activated during the process of DCIS development.

Although the underlying mechanism of SQLE dysregulation in BC remains unclear, SQLE is frequently altered by copy number gains in BC, and SQLE expression appears to be tightly regulated by increases in the copy dosage of its gene locus (17,24,26). SQLE methylation has also been associated with SQLE overexpression in BC (25).

Recently, it has been suggested that the dysregulation of SQLE may be associated with the prognosis in patients with BC. Helms *et al* (17) compared SQLE mRNA expression of BC cases with and without chromosomal 7p and 8q gains by suppression subtractive hybridization PCR. SQLE mRNA expression was upregulated in BC with ER+ 7p/8q gains (17). Although SQLE expression was not associated with tumor size, grade and ER or HER2 status, SQLE mRNA expression was an independent risk factor for the early onset of distant metastasis among early stage I/II BC cases (17). Simigdala *et al* (20) found that the cholesterol biosynthesis pathway was the common adaptive mechanism associated with acquired resistance to aromatase inhibitors in the ER+ long-term estrogen-deprived cell lines. The aforementioned study analyzed *in-silico* data from primary patients with ER+ BC with neoadjuvant aromatase inhibitor therapy and revealed that increased on-treatment SQLE expression was significantly associated with poor response to endocrine therapy (20). Kim *et al* (21) found that SQLE was one of the important genes for BC metastasis based on a standardized pathway-based approach. Shkurnikov *et al* (22) searched for novel parameters predicting the risk of relapse in patients with BC using public microarray datasets, revealing that SQLE expression was significantly associated with the risk of relapse in patients with BC. Parada *et al* (23) examined racial differences in the expression levels of eight genes, including SQLE, and their associations with the risk of BC recurrence among white and black women with BC. Compared with white women, black women exhibited higher expression levels of SQLE, and high SQLE expression was associated with increased risk of BC recurrence (23). Chin *et al* (24) performed a high-resolution comparative genomic hybridization analysis in BC, revealing that 8q24 locus, where the

SQLE gene resides, is one of the hotspot genomic regions exhibiting the strongest association between copy number gain and aberrant gene expression in high-grade, ER⁻ BC. Furthermore, amplification of 8q24 was associated with a poor prognosis independently of standard prognostic factors (24). Brown *et al* (26) independently confirmed that SQLE is *a bona fide* oncogene by amplification with clinical relevance in BC. SQLE overexpression was more prevalent in high-grade, HER2⁺ and hormone receptor-negative invasive BC, and was an independently significant unfavorable prognostic biomarker in BC (26). Yu *et al* (18) evaluated gene expression profiles from early-onset BC tissues (age of patients, <40 years) and their adjacent normal tissues to explore the genes and prognostic factors associated with BC, revealing that SQLE expression was upregulated in BC and that high SQLE expression was associated with a poor prognosis.

Most studies on SQLE in BC have been based on molecular approaches. The present study investigated whether SQLE detection by immunohistochemistry could predict the prognosis in patients with BC. High SQLE expression was more prevalent in aggressive BC, such as larger tumor size, nodal metastases, higher stage, HER2 subtype and distant metastatic relapse. High SQLE expression was associated with poor disease-free survival and overall survival, and independently predicted unfavorable disease-free survival in patients with BC. SQLE was subsequently verified as a prognostic biomarker for BC using the public BreastMark database. High SQLE mRNA expression was significantly associated with a poor prognosis in the 'all', lymph node negative, lymph node positive, luminal A subtype and luminal B subtype groups. The present results support the findings of previous studies (17,18,20-24,26) and suggest that high SQLE expression assessed by immunohistochemistry may be associated with a more aggressive phenotype in BC and may be used as a prognostic marker in patients with BC.

In the present study, high SQLE expression in DCIS was significantly associated with high nuclear grade, comedo-type necrosis and HER2 positivity, which are risk factors for DCIS recurrence or progression (27). However, high SQLE expression was not associated with the recurrence of DCIS. Considering the potential prognostic value of SQLE expression in patients with DCIS, further studies in a large cohort of DCIS cases with long-term follow-up period are required.

Several studies have demonstrated that SQLE promotes cancer cell proliferation and migration, and the presence of SQLE inhibitors in both *in vitro* and *in vivo* models causes cancer cell death (11,13). Brown *et al* (26) demonstrated that SQLE inhibition decreased the viability of a BC cell line in a copy-dosage-dependent way and increased the doubling time only in BC cell lines with high SQLE expression. In the present study, SQLE mRNA expression was significantly increased in BC tissues compared with in adjacent normal breast tissues. The current findings may pave the way for the development of novel therapeutic strategies aimed at SQLE in BC. Further studies *in vivo*, such as animal models of BC, are required to elucidate the mechanism of action of SQLE in promoting BC and to evaluate the therapeutic efficacy of SQLE inhibitors for the treatment of BC.

The selection of appropriate internal control genes is crucial for proper interpretation of RT-qPCR data. In the present

study, only the GAPDH gene was used as an internal reference control because the mRNA expression levels were constant in different tissue samples. It is recommended to use at least two reference genes to increase the resolution and accuracy of the RT-qPCR analysis (37). Therefore, the accuracy of the RT-qPCR results in the present study may be limited.

In summary, the current results suggested that upregulation of SQLE expression serves an important role in BC progression. Analysis of SQLE expression by immunohistochemistry may be a useful biomarker to predict the prognosis in patients with BC. Therefore, the present findings may open the way for further research in clinical settings to assess the relevance of SQLE inhibition as a new treatment option in patients with BC.

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to privacy and other restrictions, but are available from the corresponding author on reasonable request.

Authors' contributions

NIK conceived the experiments and wrote the manuscript. NC designed the experiments. MHP prepared the samples. NIK and MHP performed the experiments and confirmed the authenticity of all the raw data. NIK and NC processed and analyzed the data. SSK designed the study, performed statistical analysis and edited the manuscript. JSL developed the project, collected and analyzed the data, and wrote and revised the manuscript with input from all authors. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was a retrospective study that utilized archived materials of normal, DCIS and BC tissues and did not impact patient care; hence, approval was granted by the Institutional Review Board of Chonnam National University Hwasun Hospital (Jeollanam, Republic of Korea) without patient consent (reference no. CNUHH-2020-056).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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