RESEARCH ARTICLE

SLC35B2 Expression is Associated with a Poor Prognosis of Invasive Ductal Breast Carcinoma

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Abstract

Background: Breast cancer is the most common malignancy in women worldwide, including Thailand, and is a major cause of mortality and morbidity, despite advances in diagnosis and treatment. Novel gene expression in breast cancer is a focus in searches for prognostic biomarkers and new therapeutic targets. Materials and Methods: The mRNA expression of novel *B4GALT4*, *SLC35B2*, and *WDHD1* genes in breast cancer were examined in invasive ductal breast carcinoma (IDC) patients using quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR). Results: Among these genes, increased expression of *SLC35B2* mRNA was significantly associated with TNM stage III + IV of IDC (p<0.001). Hence, up-regulation of *SLC35B2* may serve as a prognostic biomarker for poor prognosis, and is also a potential therapeutic target in breast cancer.

Keywords: Invasive ductal breast carcinoma - poor prognosis - *SLC35B2*

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Introduction

Breast carcinomas are highly heterogeneous in their morphology, biology, response to therapy and clinical course. They are the most common cancers among women in Thailand (Khuhaprema et al., 2006). The trend in incidence rate of Thai women breast cancer continues to increase over time, especially in the central region (Sriplung et al., 2006), although the other country in mainland South-East Asia has a relatively low (Moore et al., 2008).The incidence of breast cancer in Thailand is 30.7/100,000/year and the death rate is 10.8/100,000/year (Ferlay et al., 2010). Thai women who have a relatively high incidence were at age of 40 years and older (Kotepui and Chupeerach, 2013) and who have lifetime occupation in an industrial (Ekpanyaskul et al., 2010). The most common pathological type of Thai breast cancer is invasive ductal carcinoma (Khuhaprema et al., 2010) with the luminal-A subtype (Chuthapisith et al., 2012).

The prognosis for breast cancer depends on its stage, typically defined as stage I to IV, with sub-stages (Taneja et al., 2010). The complexity and heterogeneity of carcinogenesis is also due to the activation of oncogenes, loss of function of tumor suppressor genes, and dis-regulation of crucial cell-signaling cascades responsible for cell growth, differentiation, communication, and apoptosis (Pakkiri et al., 2009). Several genetic alterations are reportedly biomarkers for prognosis of sporadic breast cancer, including *ERBB2* (HER2/neu), *MYC*, and *CCND1* (Cyclin D1) (Kenemans et al., 2008). Over-expression of *ERBB2* was associated with more aggressive breast-cancer characteristics (Blackwell et al., 2010) and was also used as a target for breast-cancer therapy (Yu and Hung, 2000). *MYC* amplification has been associated with poor prognosis, and is involved with *ERBB2* amplification (Nair et al., 2013). Breast-cancer patients with *MYC*/*ERBB2* co-amplification had a worse prognosis than patients who had amplified levels of only one of these (Al-Kuraya et al., 2004). High levels of *CCND1* expression have been associated with poor prognosis, particularly in ER-positive tumors, and also influenced therapeutic decisions in ER-positive breast cancer patients (Eeckhoute et al., 2006). Careful evaluation of these biomarkers with current treatment modality is required for determination. However, different breast cancer pathways emerge early in the process of oncogenesis, leading to clinically different tumor types. Therefore, it is necessary to identify novel prognostic and predictive biomarkers for breast tumors; this remains a long-awaited priority to enhance treatment.

A previous study of the gene expression profiles of Thai sporadic-breast-cancer patients indicated that the over-expression of 3 genes--UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 4 (*B4GALT4*); solute carrier family 35 (adenosine 3'-phospho 5'-phosphosulfate transporter 1) number B2 (*SLC35B2*) and WD repeat and HMG-box DNA binding protein

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1 (*WDHD1*)--was associated with advanced stages of IDC (Arnutti et al., 2013). In this study, the differential expressions of these mRNAs in IDC were verified by QRT-PCR. The association between the expression of these mRNAs and clinical outcomes was evaluated.

Materials and Methods

Patients and clinical features

A total of 35 fresh breast carcinomas and their corresponding normal tissues were obtained from the Pathology Division, Army Institute of Pathology, Phramongkutklao Medical Center, Bangkok, Thailand, between the years 2006 and 2010. No cancer patients had undergone chemotherapy or radiation before undergoing surgery. This study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand (MUTM 2013-029-01).

Sample processing

After resection, tissue samples were snap-frozen in TRIzol® reagent (Invitrogen, CA, USA) and stored at -80ºC until use. All tumor tissue sections were stained with hematoxylin and eosin, and were then analyzed by an experienced breast pathologist. Eligible samples contained >90% tumor cells. Total RNA was isolated by TRIzol® reagent (Invitrogen, USA) according to the manufacturer's instructions. The total amount of RNA isolated was quantified using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) OD_{260} measurements, and its quality evaluated by visualizing specific bands (18S and 28S rRNA) using 1.5% gel electrophoresis. First-strand cDNA was performed using Superscript® VILO™ cDNA Synthesis kits (Invitrogen, USA) and purified by DNAclear™ cDNA Purification Kit (Applied Biosystems, USA). The quantity of purified cDNA was measured by Nanodrop 1000 spectrophotometer and qualified using conventional PCR with *β-actin* primer. mRNA expression in each sample was measured by QRT-PCR.

Quantitative real-time reverse transcription polymerase chain reaction

Primers were designed by Primer-BLAST program (NCBI) using nucleotide sequences from the NCBI database. The nucleotide sequences of all primers are shown in Table 1. The QRT-PCR analysis was performed with LightCycler® FastStart DNA Master SYBR Green I (Roach, Germany). Each 20 µl of reaction mixture contained 2 µl of 10X LightCycler® FastStart DNA Master SYBR Green I, 1 µl of 5 µM forward primer, 1 µl of 5 µM reverse primer, 1.6 µl of 25 mM $MgCl_2$, 12.4 µl of sterile distilled water and 2 µl of 10 ng cDNA. The reactions were carried out using a Roche Lightcycler® 2.0 Real-Time PCR system (Roche, Germany), with the following cycling conditions: pre-incubation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing temperature (specific to the primer) for 5 seconds, and extension at 72°C for 20 second.

Analysis of gene expression using the $2^{-\Delta\Delta CT}$ method

The $2^{-\Delta\Delta CT}$ method was used to calculate relative quantification in gene expression determined by QRT-PCR, according to a previously described method (Ginzinger, 2002). The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression determined from QRT-PCR experiments. In this study, data were presented as fold-change in target genes *B4GALT4*, *SLC35B2*, and *WDHD1* expression in tumors normalized to the internal control gene (β-actin) and relative to the normal control (matched with normal as a calibrator). Over-expression of mRNA was specified as N-fold change ≥2.0, normal mRNA expression was specified as N-fold change (range 0.5001-1.9999), and under-expression was specified as N-fold change ≤0.5. All samples were screened at least twice.

Statistical analysis

Categorical data were expressed as frequency (percentage) and continuous data as mean±SD. Associations between mRNA expression and several clinic-pathological parameters, i.e., age, tumor–node–metastasis (TNM) stage, and triple-negative breast cancer (TNBC) status were analyzed using univariate logistic regression. Statistical analysis was performed using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA), and a p value of <0.05 was considered statistically significant.

Results

Clinical characteristics of the patients

Thirty-five invasive ductal carcinoma cases were initially collected for study in this investigation. The clinical outcomes of the patients and the expression profiles of 3 mRNAs are presented in Table 2. The median age of all cases was 57 (range 34-91) years. Twelve cases (34%) were aged ≤50 years and 23 (66%) >50 years. The TNM staging system, based on The American Joint

Table 1. Primer Sequences Used for QRT-PCR Amplification

Gene	Location	Primer sequence (5'-3')	Product size (bp)
β-actin	7p22	F: TCACCCACACTGTGCCCATCTACGA R: CAGCGGAACCGCTCATTGCCAATGG	295
B4GALT4	3q13.3	F: CATCCCAAGCATCTGGTGGT R: TCCCCATCCCCAGTAGTTGT	141
SLC35B2	6p12.1-p11.2	F: ACAGGGCTCCAGGTGTCTTATC R: TGCCAGCACTCGGTTCATTAGC	141
WDHD1	14q22.2	F: TCCGTTGTGTGGAACTTGCT R: GGTTGCTGTCAATTCGGCTG	143

F = forward, R = reverse, bp = base pair

Table 2. Clinicopathological Parameters and mRNA Expression Patterns of *B4GALT4*, *SLC35B2* and *WDHD1* of Intraductal Breast Carcinoma Patients

No.	Age	TNM stage*	TNBC**	mRNA expression patterns***		
				B4GALT4	*SLC35B2*	*WDHD1*
1	76	IIIA	–	–	+	–
2	80	II	–	–	n	TUD
3	70	IIIC	–	NUD	+	–
4	45	IIA	–	–	–	n
5	53	I	–	n	–	+
6	54	IIB	–	+	+	TUD
7	83	IIB	–	NUD	+	–
8	37	II	–	+	n	–
9	91	I	–	NUD	+	TUD
10	44	IIIA	–	n	+	NUD
11	41	IIB	–	n	–	NUD
12	38	IIIC	+	TUD	+	–
13	68	IIA	+	n	+	TUD
14	54	IIA	–	NUD	n	–
15	48	IIB	+	NUD	+	–
16	59	IIB	–	n	+	–
17	44	IIA	–	NUD	n	–
18	65	II	–	+	n	TUD
19	51	I	–	–	n	NUD
20	71	I	–	n	n	TUD
21	39	I	–	NUD	n	n
22	45	IIIA	–	+	+	NUD
23	52	I	–	n	n	–
24	66	IIIA	–	n	+	n
25	58	IIIB	–	–	+	NUD
26	78	IIA	+	+	n	NUD
27	39	I	–	NUD	–	n
28	67	IIB	–	n	n	+
29	54	IIB	–	n	n	n
30	46	IA	+	n	+	–
31	69	II	–	n	n	TUD
32	34	IIIA	–	n	+	–
33	59	IIA	–	NUD	+	–
34	61	I	–	–	n	–
35	60	IIIA	–	n	+	NUD

*TNM stage = tumor–node–metastasis stage; **TNBC= triple-negative breast cancer (Estrogen receptor, Progesterone receptor, Human epidermal growth factor receptor 2) (+ triple negative, - non-triple negative); ***mRNA expression patterns (+ = increased, - = decreased, n = normal, TUD = tumor undetectable, NUD = normal undetectable at 45 cycles of QRT-PCR)

Committee on Cancer (AJCC) system, identified 26 (74%) patients with TNM stage I and II tumors, and 9 (26%) with TNM stage III and IV. The percentages of patients with non-triple negative and triple-negative breast cancer were 86% (30 cases) and 14% (5 cases), respectively.

Differential gene expression

QRT-PCR SYBR Green based dye I detection was performed to determine the mRNA signatures for these 3 genes in the 35 tumor sample and their normal counterparts, using the *β-actin* gene as an endogenous reference gene. Differences in *B4GALT4*, *SLC35B2*, and *WDHD1* gene expression correlated with TNM stage. The results indicated that increase of *B4GALT4*, *SLC35B2* and *WDHD1* mRNA expression was observed in 5 of 35 cases (14%), 17 of 35 cases (49%) and 2 cases (6%), respectively. Notably, the over-expression of *SLC35B2* was observed in all the patient with TMN stage III+IV (9 cases), while 8 of 26 cases with stage I+II harbored up regulation of this gene (Figure 1). Likewise, the clinical data indicated that 4 patients present tumor staging IIB while the other 4 case harbored stage I+IIA with triple negative characteristic. Univariate logistic regression analysis showed that increased levels of *SLC35B2* mRNA expression correlated with TNM stage III+IV of IDC (P < 0.001) (Table 3).

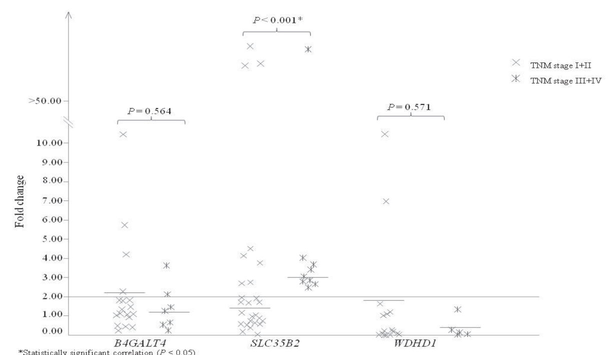

Figure 1. Differential Gene Expression of the Candidate Genes Correlated to the TNM Stage of Breast Cancer. Expression patterns of *B4GALT4*, *SLC35B2* and *WDHD1* mRNA in IDC were diagnosed by QRT-PCR using *β-actin* mRNA as a internal control. The scatter plots was presented the N-fold differential expression in the target gene correlated with TNM stage of IDC. The over-expression were defined by at least 2-fold

Table 3. Univariate Logistic Regression Analysis of *B4GALT4*, *SLC35B2* and *WDHD1* mRNA Expression and Clinicopathological Parameters in Breast Cancer

Parameters	*B4GALT4* Increased mRNA expression (n=25)				*SLC35B2* Increased mRNA expression (n=35)				*WDHD1* Increased mRNA expression (n=21)			
	Yes, n (%)	No, n (%)	OR (95%CI)	p value	Yes, n (%)	No, n (%)	OR (95%CI)	p value	Yes, n (%)	No, n (%)	OR (95%CI)	p value
Age at diagnosis												
≤50	2(29)	5(71)	2.000	0.436	6(50)	6(50)	1.091	0.592	0(0)	9(100)	-	0.314
>50	3(17)	15(83)	(0.256-15.623)		11(48)	12(52)	(0.270-4.408)		2(17)	10(83)		
TNM stage												
I+II	4(22)	14(78)	1.714	0.564	8(31)	18(69)	–	<0.001*	2(12)	14(88)	-	0.571
III+IV	1(14)	6(86)	(0.157-18.726)		9(100)	0(0)			0(0)	5(100)		
TNBC												
-	4(18)	18(82)	2.250	0.504	13(43)	17(57)	5.231	0.151	2(11)	16(89)	-	0.729
+	1(33)	2(67)	(0.162-31.329)		4(80)	1(20)	(0.521-52.551)		0(0)	3(100)		

*Statistically significant correlation (p<0.05); **OR=odds ratio; CI=confidence interval; TNM stage=tumor–node–metastasis stage; TNBC=triple-negative breast cancer (+triple-negative, -non-triple negative)

Discussion

Breast cancer is still a potentially detrimental or even fatal cancer among women, despite developments in therapeutic techniques. Early diagnosis and therapeutic treatment of breast cancer leads to reduced progression of disease and is associated with a lower rate of mortality (Berry et al., 2005). Conventional prognostic factors include axillary lymph node status, tumor size, hormone-receptor status (ER and PR), expression of HER-2, and histological grade (Fitzgibbons et al., 2000). Other clinicopathological parameters and new molecular markers are under investigation, to improve the predictability of clinical characteristics. Two of these factors, axillary lymph node status and tumor size, have been categories in the TNM staging system designated by the American Joint Committee on Cancer (AJCC) staging system (Singletary et al., 2002; Edge and Compton, 2010). The TNM staging system is an internationally accepted system used to determine disease stage, and is defined as tumor stages I through IV. Tumor stage provides information about the extent of disease, which is used to aid in personal prognosis and guide therapeutic decisions. Moreover, the TNM staging system provides a framework for reporting therapeutic outcomes and thereby permits the efficacy of new treatments to be assessed (Woodward et al., 2003). Chances of survival decrease for each successive stage of breast cancer. 88 percent of women diagnosed with stage I breast cancer survive at least five years beyond their diagnosis, whereas 5-year survival rates for stage II, III, and IV cancers are 60-80%, 40-50%, and 15%, respectively (Reed et al., 2000; Frkovic-Grazio et al., 2002; Olivotto et al., 2003). In practically, expression of estrogen receptor (ER), progesterone receptor (PR) and HER-2 are used to identify aggressiveness of breast cancer. Over-expression of HER-2 is associated with advanced clinical stages, high rate of ER/PR double negative and poor survival in breast carcinomas (Jana et al., 2012; Liu et al., 2012). Moreover, the lacking of these three receptors is classified as triple-negative breast cancer (TNBC). The comparison of 7 years and 9 years disease-free and overall survival rates between TNBC patients and non-TNBC patients reported that the TNBC are correlated with younger disease onset age, larger tumor size, higher rate of axillary lymph node positivity, and higher tumor histological grade (Li et al., 2013). Furthermore, The TNBC is linked with a poor clinical outcome, frequent relapses and metastasis (De Giorgi et al., 2007; Ismail-Khan and Bui, 2010).

Improving the understanding of gene expression, profiling, and innovative molecular analysis technology, are all steps needed to identify predictive and prognostic genes, which could help characterize tumors and enable better-tailored therapies (de Snoo et al., 2009; Kuderer and Lyman, 2009). Many of the molecular markers studied have both prognostic and predictive values. Generally, the classical molecular markers consist of *Ki67*, *ER*, *PR*, and HER2, while novel molecular markers include *p53*, *p14ARF*, *cyclin D1*, *cyclin E*, *TBX2/3*, *BRCA1/2*, and *VEGF* are involved with the development of human breast cancer (Taneja et al., 2010). Moreover, various gene such as *KAI1*, *KISS1* and *EMSY* have been reported for the correlation to poor prognosis of breast cancer (Zhang and Jin, 2010; Madjd et al., 2014) whereas, increase mRNA expression of *LPHN3* and *MMP13* were associated with axillary-node metastasis (Kotepui et al., 2013). The use of classical markers to predict patient survival and therapeutic response to breast cancer has been well established, and they continue to be used as useful laboratory tests. Although abundant genetic and phenotypic alterations have been reported in breast cancer, only a handful of these have been identified and brought to clinical studies.

The solute carrier family 35 (adenosine 3'-phospho 5'-phosphosulfate transporter 1), member B2 gene (*SLC35B2*) located in 6p12.1-p11.2 and encodes the protein adenosine 3'-phospho 5'-phosphosulfate transporter 1 (PAPST1). The PAST1 protein is one of two putative PAPS transporters; another is PAST2 (SLC35B3). Its function is the transportation of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) from the cytosol, where it is synthesized, by a bifunctional PAPS synthetase (Li et al., 1995), into the Golgi lumen (Kamiyama et al., 2003). Sulfation is essential for the post-translational alterations of glycoproteins, proteoglycans, and glycolipids required for normal growth and development. The PAPS or nucleotide sulfate is a universal sulfuryl donor for sulfation (Kamiyama et al., 2003). Sulfate is transferred from PAPS to a defined position on the sugar residue by sulfotransferases. Two PAPS transporter genes have been identified in both humans and Drosophila (Kamiyama et al., 2003; Luders et al., 2003; Goda et al., 2006). In Drosophila, these PAPS transporters are required for the sulfation of cellular proteins and normal development. In humans, both PAPST1 (*SLC35B2*) and PAPST2 (SLC35B3) are necessary for the sulfation of the 6-sulfolactosamine epitope in a human colorectal carcinoma cell line (Huopaniemi et al., 2004; Kamiyama et al., 2006). The *SLC35B2* gene also plays an important role in cancer-cell proliferation, by controlling their sulfation status as part of a desmoplastic reaction to support cancer growth in colorectal cancers (Kamiyama et al., 2011). Furthermore, over-expression of PAPST 1 or PAPST 2 reduced radiation-induced apoptosis in human Burkitt's lymphoma cells (Nakayama et al., 2013). This study showed that increased levels of *SLC35B2* mRNA in invasive ductal breast carcinomas are associated with advanced-stage breast cancer. Thus, *SLC35B2* could be involved with a poor prognosis in human breast cancer. In light of this, the role of *SLC35B2* deserves further study, to help determine the genetic factors affecting prognosis and treatment of IDC.

In summary, QRT-PCR was used to validate the prognostic gene profile in Thai breast-cancer patients obtained from microarray data. The results suggested that up-regulation of *SLC35B2* is associated with a poor prognosis among patients with invasive ductal breast carcinoma. Interestingly, this may be the first report of the increase in *SLC35B2* mRNA expression associated with a poor prognosis in breast cancer. Consequently, *SLC35B2* may be a potential candidate prognostic biomarker in breast cancer patients, as well as a potentially selective

therapeutic target. Further studies with larger sample sizes will increase the precision of the data.

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100.0
75.0
50.0
25.0
0
6.3
56.3
31.3
Newly diagnosed without treatment