Abstract. The purpose of this study was to estimate the role of the SLC34A2 gene in breast cancer. A total of 146 samples were collected from breast cancer tissues and their adjacent normal breast tissues. Reverse transcription and real-time polymerase chain reaction were used to estimate gene expression levels. There was a significantly increased gene expression of SLC34A2 (normal tissues: 6.71±0.77; tumour tissues: 10.29±0.80) among breast cancer tissues compared with normal tissues. However, there was no significant association between overall survival and the gene expression level of SLC34A2. Moreover, a significant overexpression of CA125 (normal tissues: 7.26±0.62; tumour tissues: 10.51±0.58) in breast cancer tissues and a significant correlation between SLC34A2 and CA125 gene expressions were found. Our results suggested SLC34A2 to be involved in the development of breast cancer; this gene may therefore be a novel marker for the detection of breast cancer and act as a target gene in therapeutic strategies.

Breast cancer is an epithelial tumour with highly invasive and metastatic potential and is one of the most frequently occurring malignant neoplasms worldwide (1, 2), as well as the fourth leading cause of cancer death among Taiwanese people in Taiwan (3). Thus, there is a demand for novel treatment modalities in breast cancer, such as approaches to targeted therapies. Recently, hormone-related factors, such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal receptor 2 (HER2) (4-7), and screening of gene expression, such as CA125 (8-11), have been considered to be associated with the development and prognosis of breast cancer and suggested to be used as predictors of, or in the targeted treatment of, breast cancer. However, the lack of a specific biomarker for CA125 (10, 12-17), and the controversial role of hormone-related receptors (4-7) for breast cancer have limited application in breast cancer prediction or targeted therapy. Therefore, additional biological and clinical studies on their significance in the diagnosis and therapy of breast cancer are required.

The SLC34A2 gene, located on chromosome 4p15.2, is a member of the solute carrier gene family, which encodes for a multi-pass membrane protein of 690 amino acids. SLC34A2 is expressed on cell surfaces as a heavily glycosylated plasma membrane protein for mediating the transport of inorganic phosphate into epithelial cells via sodium ion co-transport (18-20), and is suggested to be associated with calcification in several tissues (18, 19, 21). Increased inorganic polyphosphate has been reported to promote the proliferation of human fibroblasts and human dental pulp cells (22), and calcium-phosphate calcifications.
are thought to be involved in the development of breast cancer (23-26). This transporter has also been considered to be associated with cell differentiation (27) and may play a role in tumourigenesis (28). An increased expression of SLC34A2 has been reported for ovarian cancer (12) and papillary thyroid cancer (29), but a significantly decreased expression has been demonstrated for human non-small cell lung carcinomas (28).

In this study, it was hypothesised that overexpression of SLC34A2 might be associated with breast cancer development and metastasis, and that it is possibly a target for breast cancer therapy.

Materials and Methods

Subjects and specimen collection. Seventy-three patients with breast-infiltrating ductal carcinoma who underwent surgical treatment at Changhua Christian Hospital between 2002 and 2007 were recruited for this study. One hundred and forty-six tissue specimens, collected from breast cancer tissues and their adjacent normal breast tissues, were frozen immediately in liquid nitrogen and stored at –80˚C until further analysis. The histological types of the primary tissues and the clinicopathological stage of the breast cancer were both determined by a single pathologist, according to a system based on a modification of the WHO classification and the TNM system (30), respectively. Associated clinicopathological characteristics, such as the clinical stage of breast cancer, cell differentiation status, lymph node metastasis, and distant metastasis, were verified by chart review.

Tissue RNA extraction. Total RNA was extracted from frozen breast tissues using RNA-Bee™ (Tel-Test, Friendswood, TX, USA), following the manufacturer’s instructions. Tissue samples were homogenised in RNA-Bee™. Chloroform was added per 1 ml of RNA-Bee, the mixture was vigorously shaken for 15-30 minutes, and then centrifuged at 4˚C. The aqueous phase was transferred to a clean tube, isopropanol was added, and the samples stored overnight at –20˚C. The samples were then centrifuged at 12,000 xg for 5 minutes at 4˚C. The RNA precipitate formed a white-yellow pellet at the bottom of the tube. The supernatant was removed and the RNA pellet was washed once with 75% ethanol, and then was dissolved in diethyl pyrocarbonate (DEPC)-H2O and stored at –80˚C until reverse transcription.

Generation of complementary DNA (cDNA). Total RNA was used to prepare cDNA using RT-PCR. The reaction mixtures contained RNA, oligo(dT)15 (Promega, Madison, WI, USA) and random primer (Protech, Taipei, Taiwan); the volume was adjusted with DEPC-H2O, heated at 70˚C for 5 minutes, and placed on ice for 3 minutes. Then, reverse transcriptase mixtures which contained MMLV (Protech), dNTPs (Protech), DTT (Protech), and RNasin (Protech) were added. The reaction was incubated at 37˚C for 4 hours. The concentration of cDNA was measured by spectrophotometer BioPhotometer 6131 (Eppendorf, Germany) and adjusted to 150 ng/ml for real-time PCR.

Quantitative real-time PCR analysis. The SLC34A2 and CA125/MUC16 genes were examined quantitatively for expression levels by real-time PCR using the TaqMan probe real-time PCR assay (Roche Diagnostic, Germany). Homo sapiens ribosomal protein S18 was used in the study as an endogenous control gene. Two of the most commonly used endogenous control genes for breast cancer gene expression studies are glyceraldehyde-3-phosphate dehydrogenase and β-actin. The following primer pairs were used for SLC34A2 and CA125/MUC16 gene transcripts: SLC34A2 (forward primer, 5’-ATCAAGAAGACCATCAACT-3’; reverse primer, 5’-GGTATGCGATTACA-3’; amplicon length 155 bp), CA125/MUC16 (forward primer, 5’-TTCGAAACAGCAAGCTCA-3’; reverse primer, 5’-GGGAGAATCCCATCCACA-3’; amplicon length 234 bp).
The amplification of the target genes was as follows: 95°C for 10 minutes, followed by 30 cycles of 95°C for 15 seconds and 58°C for 1 minute. Triplicate experiments were carried out for each pair of RNA samples to minimize technical variations and for statistical analysis.

**Immunohistochemical analysis.** The protein expression of ER, PR, and HER2 in cancerous tissue was analysed based on standard immunohistochemistry. Immunostaining was performed with anti-ER (Dako, ID5), anti-PR (Dako, PGR 636), and anti-HER2 (Dako, Carpinteria, CA, USA), respectively, using an autostaining and semiquantitative scoring system (Ventana, Arizona, USA). All hormone-related receptor statuses were evaluated according to breast pathology guidelines. Estimation of ER and PR protein expression was carried out by analysis in which a value less than 10% of expression was considered as negative staining for hormone receptor, and an expression level greater to or equal to 10% of expression was considered as positive staining. An expression level higher than 10% and less than 50% was scored grade 1; between 50% and 75% was scored grade 2, and higher than 75% was scored grade 3. In the assessment of the staining of HER2, no staining or membrane staining of less than 10% of invasive tumour cells was considered as negative staining and scored grade 0; a barely perceptible membrane staining detected in more than 10% was also considered as negative staining and scored grade 1; a weak to moderate complete membrane staining in more than 10% or less than 30% with strong complete membrane staining was considered an equivocal staining and scored grade 2; a strong complete membrane staining in more than 30% was considered as positive staining and scored grade 3. In grade 2+ cases, fluorescent in situ hybridisation was performed and the HER2 status was judged on that basis.

**Statistical analysis.** Experimental results are presented as the mean±SE. A nonparametric method was used since the distribution of experimental results was not normal for some variables. A Wilcoxon signed-ranks test was used for evaluation of gene expression in normal and tumour tissues of patients with breast cancer. The comparisons of absolute increased gene expression levels between two compared clinical statuses was estimated using the Mann-Whitney U-test. The correlations were examined with Spearman’s rank correlation. The Kaplan-Meier curve model was used for univariate analysis to identify factors most significantly related to overall survival (December 31, 2008). A p-value <0.05 was considered significant. The data were analysed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA) statistical software.

**Results**

The mean age (±SE) of those 73 recruited patients with breast cancer was 58.68±1.50 years. The gene expression profiles of SLC34A2 and CA125 were determined in breast cancer tissues and their adjacent normal breast tissues. There were significantly increased gene expressions (mean±SE) of SLC34A2 (normal tissues: 6.71±0.77; tumour tissues: 8.70±0.80).
10.29±0.80; p=0.001; Figure 1A) and CA125 (normal tissues: 7.26±0.62; tumour tissues: 10.51±0.58; p<0.0001; Figure 1A) among breast cancer samples compared with their adjacent normal breast tissues.

The comparisons of absolute increased gene expression levels of SLC34A2 between two compared clinical statuses were estimated by subtracting the gene expression levels of normal tissues from that of tumour tissues, as shown in Figure 2. The absolute increased gene expression levels (mean±SE) of SLC34A2 were 3.69±1.10 in clinical stage ≤II, 3.83±2.06 in clinical stage ≥III, 3.27±1.23 in well to moderate cell differentiation, 4.64±1.56 in poor cell differentiation, 3.49±1.25 in non-lymph node metastasis, 4.06±1.56 in lymph node metastasis, 4.03±1.06 in non-distant metastasis, and 1.84±2.30 in distant metastasis of each separate group, respectively. There was no significant difference in absolute increased gene expression levels between each paired comparison for the SLC34A2 gene.

There was a significant correlation (r=0.407; p=0.0001) between CA125 and SLC34A2 gene expressions in breast tumour tissues was found using Spearman’s rank correlation.

There was a significant correlation (r=0.407; p=0.0001) between CA125 and SLC34A2 gene expression in breast cancer tissues (Figure 3). However, there were no correlations between the gene expression of SLC34A2 and those of ER (r=0.075, p=0.52), PR (r=0.065, p=0.59), and HER2 (r=0.13, p=0.25) protein expression in breast cancer tissues (data not shown).

During a mean follow-up of 53.8 months (range, 8.4-64.5), 11 (15.1%) of the 73 patients had died. Overall survival was 84.9% among 73 recruited patients, and in the subgroup, overall survival was 86.7% and 82.1% in the over- and non-over-expression of SLC34A2 subgroup, respectively. Using the Kaplan-Meier method, it was found that there was no significantly difference in overall survival (p=0.55) between the over- and non-overexpression subgroups of SLC34A2 (Figure 4).

**Discussion**

In this study, it was hypothesised that overexpression of SLC34A2 might be associated with the development of breast cancer, and it was found that the SLC34A2 gene expression was significantly increased in breast cancer tissues, compared to their adjacent normal breast tissues. These results are similar to those of Rangel et al. (12) and Jarzab et al. (29).

A previous study demonstrated that calcium phosphate crystals dramatically stimulated cellular endocytosis, and that this endocytic activity was suggested to be associated with the alteration of molecular signal transduction (31). Moreover, calcium hydroxyapatite was reported to enhance human breast cancer cell line mitogenesis (24), and promote growth hormone release (32). Overexpression of SLC34A2 can increase the reabsorption of inorganic phosphate, which contributes to the production of calcium phosphate-mediated calcifications in breast tissue, and then alter cellular signal transduction or increase growth hormone release, which consequently induces proliferation and carcinogenesis of breast epithelial cells, although this mechanism needs further investigation.

Calcium hydroxyapatite has been reported to enhance the expression of matrix metalloproteinase (MMPs), including
MMP-2,-9, and -13 in MCF-7 breast cancer cell lines and MMP-9 in human mammary epithelial cell lines (24). In addition, inorganic polyphosphate has been demonstrated to increase MMP-1 expression in multipotent mesenchymal stem cells (22). In this study, it was supposed that overexpression of SLC34A2 in breast cancer tissues might be associated with cell invasion, metastasis, and overall survival of breast cancer due to its competency in increasing the absorption of phosphate. However, it was found that there was no significant difference in the absolute increase in gene expression levels of SLC34A2 between each paired comparison clinical stage, including pathological stage and the status of tumour cell differentiation, lymph node metastasis, and distant metastasis. Also, there were no significantly different overall survival distributions between the over- and non-overexpression subgroups of SLC34A2.

The role of hormone-related receptors in breast cancer is still controversial, although it has been reported that an invasive grade of breast cancer was positively correlated with HER2 expression (33, 34), but negatively correlated to ER and PR expression (35-37). In order to clarify whether over-expression of SLC34A2 in breast cancer tissues is associated with the expression levels of hormone-related receptors, the correlations between SLC34A2 gene and HER2, ER, and PR protein expression levels were estimated, and it was found that there were no correlations between SLC34A2 and these hormone-related receptor expression levels.

Increased CA125 gene expression and its application in breast cancer (8-11), as well as in other types of cancer (10, 12, 15-17), have been studied extensively; however, it has been considered that this tumour marker is usually influenced by personal or medical factors (13, 14). In this study, the CA125 gene expression level was used as a marker of breast cancer (8-11), and the comparison of CA125 gene expression between breast cancer tissues and their adjacent normal breast tissues, as well as the correlation between CA125 and SLC34A2 gene expression in breast cancer tissues, was made. The present study revealed CA125 gene expression was significantly increased in tumour tissues compared to normal tissues among the sampled breast tissues. Furthermore, a significant correlation was found between SLC34A2 and CA125 gene expression levels in tumour tissues. In summary, these results support the hypothesis that the SLC34A2 gene might be a novel marker for detection of breast cancer and a target gene for therapeutic strategies. To the best of the Authors’ knowledge, this is the first study to provide novel information regarding significantly increased SLC34A2 gene expression in breast cancer tissues compared to their adjacent normal breast tissues.

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Ethical Approval

The study was conducted with the approval of the Changhua Christian Hospital Institutional Review Board and informed written consent was obtained from each individual.

References


