High *SLC20A1* Expression Is Associated With Poor Prognoses in Claudin-low and Basal-like Breast Cancers

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Abstract. *Background/Aim:* *SLC20A1* has been identified as a prognostic marker in ER+ breast cancer. However, the role of *SLC20A1* expression in breast cancer subtypes other than the ER+ types remains unclear. *Materials and Methods:* Genomics datasets were downloaded and analyzed, and the effect of *SLC20A1* knockdown using targeted siRNA on cell viability and tumor-sphere formation was assessed. *Results:* *SLC20A1*^{high} patients with ER+, claudin-low or basal-like breast cancers showed poor prognoses. *SLC20A1*^{high} patients treated with radiotherapy had poor clinical outcomes. *SLC20A1* knockdown suppressed the viability of MDA-MB 231 (claudin-low), MDA-MB 468 (basal-like) and MCF-7 (ER+) cells, and tumor-sphere formation by ALDH1^{high} cells. These results suggest that *SLC20A1* is involved in cancer progression and contributes to clinical outcomes in patients with ER+, claudin-low and basal-like breast cancers. *Conclusion:* *SLC20A1* is a potential prognostic marker and therapeutic target in ER+, claudin-low and basal-like breast cancers.

Breast cancer is the most commonly occurring cancer among women worldwide, with 2.1 million new cases (24.2% of all

cancers in women) and 0.6 million cancer-related deaths (15.0% of all cancer-related deaths among woman) annually (1). Breast cancer is classified using immunohistochemistry (IHC) and gene expression patterns (PAM50) (2-7). Based on IHC, breast cancer is classified into four types: ER+ and/or PgR+ HER2- type, ER+ and/or PgR+ HER2+ type, ER- and PgR- HER2+ type and triple negative type (TNBC). Based on PAM50, breast cancer is classified into at least six subtypes: normal-like, luminal A, luminal B, HER2-enriched, claudin-low and basal-like (2-7). Among these, the luminal A and luminal B types express ER (8, 9), and some luminal B and HER2-enriched types express HER2 (4, 8-11). Many claudin-low and basal-like types overlap with the TNBC type (6, 12-14).

Breast cancer treatment mainly entails surgery, radiotherapy and drug therapy, which may include chemotherapy, endocrine therapy and/or molecular target therapy. Overall, breast cancer prognosis is good. Endocrine therapy is selected against ER+ type and a HER2-targeted antibody, such as trastuzumab, is used to treat HER2 type (11, 15, 16). However, there is no effective drug or molecular targeted therapy for TNBC or its overlapping claudin-low and basal-like types. Consequently, those patients are treated only with surgery, radiotherapy and chemotherapy, and have poor prognoses (6, 13, 15-17). Moreover, it is also known that in some of these cases, chemo- and/or radiotherapy actually stimulates cancer progression (18-20). It is therefore essential to identify effective prognostic markers and molecular targets that can be exploited for the treatment of the claudin-low and basal-like breast cancer subtypes.

A major hurdle that must be overcome for therapy to be effective against the claudin-low and basal-like subtypes is

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their high degree of stemness (12, 21). Tumors consist of both differentiated cancer cells and cancer stem cells (CSCs). CSCs are largely undifferentiated tumorigenic cells that exhibit stem-like functions such as self-renewal and multipotency (22, 23). Because most CSCs are resistant to conventional chemo- and radio-therapies, development of targeted therapies against CSCs is very much needed to improve the clinical outcomes of patients with these cancers (21, 22, 24). ALDH1 is an enzyme that converts aldehydes to carboxylic acids and is abundant in TNBC (25-27). Among the ALDH1 genes, *ALDH1A1* and *ALDH1A3* are known to be CSC markers in several cancers (25, 28-33). In particular, *ALDH1A1* is enriched in claudin-low type breast cancers while *ALDH1A3* is enriched in the basal-like type (34).

SLC20A1/PiTi encodes the sodium/inorganic phosphate (Pi) symporter, which was originally identified as a mammalian retroviral receptor (35) and is responsible for the uptake of Pi into cells (36). SLC20A1 is expressed in various tissues and plays a fundamental housekeeping role supplying Pi to cells (35, 36). SLC20A1 gene knockout mice exhibit hypoplastic livers accompanied by a cell proliferative defect and induced cell death that result in embryonic lethality at E12.5 (37). SLC20A1 promotes cell proliferation in murine pre-osteoblastic cells, NIH3T3 cells, primary mouse embryonic fibroblasts, human HepG2 cells and HeLa cells (37-39), and it is also involved in tumor formation by HeLa cells in xenografted mice (39). SLC20A1 is highly expressed in human somatotroph adenomas and is involved in the proliferation and migration of GH3 cells derived from somatotroph adenomas (40). We recently reported that high *SLC20A1* expression correlates with poor prognosis of ER+ breast cancer (41). However, the role of *SLC20A1* expression in breast cancer subtypes other than the ER+ type remains unknown, as does its function in ALDH1+ breast CSCs. In this report, we show that *SLC20A1* expression is a potential prognostic marker for ER+, claudin-low and basal-like breast cancers and that SLC20A1 depletion suppressed the viability of ER+, claudin-low and basal-like breast cancer cells as well as *in vitro* tumor-sphere formation by ALDH1^{high} cells.

Materials and Methods

Analysis of The Cancer Genome Atlas dataset. The Cancer Genome Atlas (TCGA) breast cancer dataset (42) was downloaded from Oncomine (43) on January 9, 2020. The clinicopathological data from these patients have been summarized previously (34). This dataset contains mRNA expression data from 61 normal breast tissue samples and 532 primary breast tumor samples. Expression of SLC20A1 mRNA (reporter: A_23_P165656) was compared between the normal and cancer tissues, and the *p*-value was calculated using the Mann-Whitney *U*-test. SLC20A1 mRNA expression was also plotted using paired comparison of normal versus cancer tissue with samples for which there were SLC20A1 mRNA data from both normal and cancer tissues from the same patients (n=60). The *p*-value was calculated using the Wilcoxon

signed-rank test. We set the level of significance at 5%, two sided. All statistical analyses were carried out using BellCurve for Excel ver. 3.00 (SSRI, Tokyo, Japan).

Analysis of the Molecular Taxonomy of Breast Cancer International Consortium dataset. The Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset (44, 45) was downloaded from the cBioportal (46, 47) on July 29, 2020. The clinicopathological data from these patients have been summarized previously (34). The METABRIC dataset contains data on both gene alterations (n=2173) and mRNA expression levels (n=1904). The mRNA expression levels were compared using the Kruskal-Wallis and Steel-Dwass multiple comparison test. We defined the optimal cutoff thresholds to divide patients into high- and low-expression groups using receiver operator characteristic (ROC) curves relating *SLC20A1* expression gene to disease-specific survival (DSS). The optimal cutoff threshold was determined using the Youden index. The χ^2 test was performed to assess the correlation between clinicopathologic parameters and *SLC20A1* expression levels. Survival curves based on DSS, were plotted using the Kaplan-Meier method, and curves were compared using the log-rank (Cochran-Mantel-Haenszel) test. A multivariate Cox regression analysis was used to evaluate the influence of gene expression and to estimate adjusted hazard ratios (HRs) using DSS statuses. We set the level of significance at 5%, two sided. All statistical analyses were carried out using BellCurve for Excel ver. 3.00 (SSRI, Tokyo, Japan).

Analysis of the DNA methylation using UALCAN. Levels of *SLC20A1* DNA methylation at the CpG island in the promoter region were analyzed using UALCAN (48). The Beta values indicate the DNA methylation level: Beta values of 0.7-0.5 indicate hypermethylation, while Beta values of 0.3-0.25 indicate hypomethylation.

Cell culture. Human claudin-low type (MDA-MB 231) and basal-like type (MDA-MB 468) breast cancer cell lines (6, 49, 50) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (Capricorn, Ebsdorfergrund, Germany). Human luminal A type (MCF-7) cells (6, 49, 50) were cultured in DMEM containing 10% FBS and 0.01 mg/ml insulin. MDA-MB 231, MDA-MB 468 and MCF-7 cells were all obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Mycoplasma testing was performed for the cell lines used.

siRNA transfection. For WST-8 assays, SLC20A1 knockdown (KD) was accomplished by transfection of two siRNAs: siRNA-1 (5'-CUC UAG UGG CUU CAG UAU UTT-3') and siRNA-2 (5'-GAA UUC GAA UGG ACA GUU ATT-3') (Sigma-Aldrich, St. Louis, MO, USA). For tumor-sphere culture, SLC20A1 KD in MDA-MB 231 and MDA-MB 468 cells was accomplished by transfection with the DsiRNA sense strand (5'-CUCUAGUGGCUUCAGUUAUUGAACTG-3') and antisense strand (5'-CAGUUCAAUACUGAAGCCACUAGAGGG-3') (IDT, Coralville, IA, USA), which results in long term suppression of gene expression (51). As controls, we used MISSION siRNA Universal Negative Control (Sigma-Aldrich) and Negative Control DsiRNA (IDT), respectively. The transfection was accomplished using siRNA mixed OPTI-MEM (Gibco, Thermo Fisher, Waltham, MA, USA) and Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen). KD efficiency was monitored using Quantitative PCR as

previously described (52) (*SLC20A1* probe, 5'-FAM/TTAGGCAACTGCACCTGCACCATTCACGG/TAMRA/-3'; forward primer, 5'-GCGTGGACTTGAAAGAGGAAAC-3'; reverse primer, 5'-CTGACGGCTTGACTGAACTGG-3').

WST-8 assay. WST-8 assay was performed as previously described (34, 53, 54). Briefly, following transfection of 10 nM siRNA, the cells were incubated for 24 h. Then, they were seeded into 96-well plates (5×10^3 cells/well) (Thermo Fisher) and incubated for an additional 24 h. Cell viability was then assessed using Cell Counting Reagent SF (Nacalai tesque, Kyoto, Japan). The formazan dye formed was measured using Sunrise Remote (TECAN, Seestrasse, Männedorf, Switzerland) at 450 nm. Statistical significance was determined with Student's *t*-test, ** $p < 0.01$, * $p < 0.05$. Data are presented as means $\pm$ SE of three independent experiments.

Tumor-sphere culture. Tumor-sphere formation was assayed as described previously (34, 52-54). Following transfection of 10 nM DsiRNA, MDA-MB 231 and MDA-MB 468 cells were cultured for 48 h. Then, ALDH1^{high} cells were isolated from the DsiRNA transfectants using an ALDEFLUOR assay kit (Stem Cell Technologies, Vancouver, British Columbia, Canada) with a FACS Aria™ III (BD Bioscience, San Jose, CA, USA). The FACS data were analyzed using Flowjo v10.6 software (BD Bioscience). The isolated ALDH1^{high} cells were plated in ultralow attachment 96-well plates (Greiner Bio-One, Kremsmünster, Austria) at a density of 1×10^3 cells/well and cultured for 7 days in MDA-MB 231 and MDA-MB 468 culture medium containing 0.6% methylcellulose (Nacalai tesque, Kyoto, Japan) and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich). For each sample, four images per well from 3 wells were randomly captured using a DMIL LED (Leica, Wetzlar, Germany). The number and size of tumor spheres over $314 \mu\text{m}^2$ were then measured using ImageJ Fiji software.

Results

SLC20A1 is more highly expressed in breast cancer than normal mammary epithelium. We first compared the levels of *SLC20A1* expression in normal breast and breast cancer tissues in TCGA breast cancer ($n=593$) dataset downloaded from the Oncomine database (43). As shown in Figure 1A, *SLC20A1* expression was higher in breast cancer than in normal mammary epithelium. Paired comparison of *SLC20A1* mRNA expression between normal and tumor tissues derived from the same patients further confirmed that *SLC20A1* expression is higher in breast cancer than normal mammary epithelium (Figure 1B).

We next examined *SLC20A1* expression among breast cancer subtypes using both TCGA and the METABRIC datasets (44, 45). Analyzed in TCGA dataset were data from normal tissues and breast cancer subtypes classified based on IHC. As shown in Figure 1C, *SLC20A1* expression was higher in all subtypes than in normal tissues. Analyzed in the METABRIC dataset were data from breast cancer subtypes classified based on IHC or PAM50. Among subtypes classified based on IHC, *SLC20A1* expression was higher in the HER2+ subtypes (ER+ and/or PgR+ HER2+ subtype, ER- and PgR- HER2+ subtype) (Figure 1D). In addition,

among subtypes classified based on PAM50, *SLC20A1* was more highly expressed in the luminal B and HER2-enriched subtypes (Figure 1E).

These results suggest that *SLC20A1* is highly expressed in breast cancer. For that reason, we assessed *SLC20A1* gene alterations using the METABRIC dataset from the cBioportal database as well as *SLC20A1* DNA methylation using UALCAN (48). *SLC20A1* gene amplification was detected in only 0.32% of breast cancers (7/2173), and other gene mutations, including deep deletion, point mutation and fusion, were undetected. In addition, the frequency of DNA methylation was lower in breast cancer than in normal tissue; Beta values, which indicate the level of DNA methylation, were very low (Normal: median, 0.048; Cancer: median, 0.045). Moreover, very little DNA methylation was detected at the CpG island in the promoter region of *SLC20A1* (Figure 1F). These results suggest the high level of *SLC20A1* mRNA expression seen in breast cancer as compared to normal tissues reflects the transcriptional activation of *SLC20A1* rather than a change in DNA methylation or gene amplification.

*Kaplan–Meier analyses indicate that clinical outcomes are poor for *SLC20A1*^{high} patients with ER+ luminal A and B types or the claudin-low or basal-like breast cancers.* Our earlier analysis of TCGA dataset ($n=526$) revealed that patients with *SLC20A1*^{high} ER+ breast cancers had poor clinical outcomes (41). However, the number of patients in the cohort was small, and the role of *SLC20A1* in breast cancer subtypes other than the ER+ type remained unknown. In the present study, therefore, to assess the role of *SLC20A1* expression in other breast cancer subtypes, we analyzed a METABRIC dataset that included the gene expression data from 1904 breast cancer patients. The relationship between *SLC20A1* expression and clinicopathologic parameters was examined using the χ^2 test, which revealed that *SLC20A1* expression did not correlate with age ($p=0.80$), tumor size ($p=0.10$), tumor stage ($p=0.36$) or lymph node metastasis ($p=0.41$). The association between *SLC20A1* expression and prognosis among the breast cancer subtypes was examined by using the Kaplan–Meier method to compare DSS between *SLC20A1*^{high} and *SLC20A1*^{low} patients. Among breast cancer subtypes classified with IHC, only *SLC20A1*^{high} patients with the ER+ and/or PgR+ HER2- subtype showed poor prognosis ($p < 0.001$; Figure 2). Among breast cancer subtypes classified based on PAM50, *SLC20A1*^{high} patients with luminal A or B, which were ER+ (luminal A: $p < 0.001$; luminal B: $p=0.026$) had poor prognosis (Figure 3C and D). Importantly, *SLC20A1*^{high} patients with the claudin-low or basal-like type also had poor prognosis (claudin-low: $p=0.0054$; basal-like: $p < 0.001$) (Figure 3F and G). On the other hand, *SLC20A1*^{high} patients with the normal-like type did not show poor prognosis ($p=0.095$) (Figure 3B), and *SLC20A1*^{high} patients with the HER2-enriched type had good prognosis ($p=0.0021$) (Figure 3E).

Figure 1. *SLC20A1* is highly expressed in breast cancer. (A-C) *SLC20A1* mRNA expression in normal mammary epithelium and breast cancer. TCGA breast data was downloaded from Oncomine. (A) Box plots showing *SLC20A1* mRNA levels in normal mammary epithelium (n=61) and all breast cancers (n=532). *** $p < 0.001$, two-sided Mann-Whitney's U test. (B) Paired comparison of *SLC20A1* mRNA expression in normal and tumor tissues from the same patients (n=60). *** $p < 0.001$, Wilcoxon signed rank test. (C) Boxplots showing *SLC20A1* mRNA levels in normal tissues and breast cancer subtypes classified based on IHC. *** $p < 0.001$, ** $p < 0.01$, Kruskal-Wallis test with Steel-Dwass test. (D, E) *SLC20A1* mRNA expression in breast cancer subtypes. For this analysis, METABRIC data was downloaded from cBioportal. (D) IHC subtypes (E) PAM50 subtypes. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ Kruskal-Wallis and Steel-Dwass multiple comparison test. (F) Box plots showing *SLC20A1* DNA methylation levels determined using UALCAN in normal (n=97) and breast cancer (n=793). The Beta values indicate the DNA methylation levels (hypermethylation, Beta values=0.7-0.5; hypomethylation, Beta values=0.3-0.25).

Figure 2. Kaplan–Meier analyses of disease-specific survival show that *SLC20A1*^{high} patients with ER+ and/or PgR+ HER2- type breast cancers have poor clinical outcomes. (A–D) Kaplan–Meier analyses comparing disease-specific survival between *SLC20A1*^{high} and *SLC20A1*^{low} patients in the METABRIC dataset: (A) ER+ and/or PgR+ HER2-, (B) ER+ and/or PgR+ HER2+, (C) ER- and PgR- HER2+, (D) TNBC subtypes. *p*-Values were calculated with the log-rank (Cochran–Mantel–Haenszel) test. *SLC20A1*^{high} patients are shown with a dark gray line, *SLC20A1*^{low} patients with a light gray line.

Multivariate Cox regression analysis confirms that *SLC20A1*^{high} breast cancer patients with the ER+ luminal A or B subtypes or the claudin-low or basal-like subtypes have poorer clinical outcomes. To confirm the results by Kaplan–Meier analyses, we performed a multivariate Cox regression analysis of DSS using the same dataset (Table I). As with the Kaplan–Meier analysis, *SLC20A1*^{high} patients with the luminal A or B subtype (luminal A: HR=1.69, 95%CI=1.21–2.37; luminal B: HR=1.65, 95%CI=1.08–2.54) or the claudin-low or basal-like subtype (claudin-low: HR=2.34, 95%CI=1.28–4.25; basal-like: HR=2.19, 95%CI=1.38–3.46) showed poorer prognosis. *SLC20A1*^{high} patients with the normal-like subtype did not show poor prognosis (HR=0.57, 95%CI=0.28–1.15), and those with the HER2-enriched subtype showed good prognosis (HR=0.44, 95%CI=0.26–0.75). These results strongly suggest that *SLC20A1*^{high} is involved in cancer progression and contributes to poor clinical outcomes in breast cancer patients with ER+, claudin-low and basal-like breast cancers.

Chemotherapy was not associated with poorer clinical outcomes in *SLC20A1*^{high} patients with claudin-low or basal-like breast cancer. Because chemotherapy is the primary treatment for claudin-low and basal-like breast cancers (15, 16), we investigated the association between *SLC20A1* expression and chemotherapy in claudin-low and basal-like breast cancer (claudin-low without chemotherapy, n=130; claudin-low with chemotherapy, n=69; basal-like without chemotherapy, n=91; basal-like with chemotherapy, n=108). Both Kaplan–Meier and multivariate Cox regression analyses showed that *SLC20A1*^{high} patients treated without chemotherapy had poorer prognosis than *SLC20A1*^{low} patients (KM: claudin-low: *p*<0.001; basal-like: *p*<0.001) (COX:

claudin-low: HR=3.73, 95%CI=1.80–7.76; basal-like: HR=4.97, 95%CI=2.39–10.36) (Figure 4A and B, Table I). By contrast, among patients treated with chemotherapy, the prognosis of *SLC20A1*^{high} patients were no poorer than those of *SLC20A1*^{low} patients (KM: claudin-low: *p*=0.54; basal-like: *p*=0.20) (COX: claudin-low: HR=0.80, 95%CI=0.34–1.86; basal-like: HR=1.39, 95%CI=0.78–2.47) (Figure 4C and D, Table I). Multivariate Cox regression analysis revealed that adding radiotherapy to chemotherapy as a covariate was associated with a poor prognosis (No chemotherapy: claudin-low: HR=3.81, 95%CI=1.84–7.92; basal-like: HR=4.86, 95%CI=2.32–10.18) (Chemotherapy: claudin-low: HR=0.79, 95%CI=0.34–1.85; basal-like: HR=1.33, 95%CI=0.74–2.37) (Table I). These results suggest that chemotherapy is an effective treatment against the claudin-low and basal-like types of *SLC20A1*^{high} breast cancer.

Radiotherapy was associated with poorer clinical outcomes in *SLC20A1*^{high} patients with claudin-low or basal-like breast cancer. Because radiotherapy is also used in the treatment of breast cancer, we investigated the association between *SLC20A1* expression and radiotherapy in patients with claudin-low or basal-like breast cancer (claudin-low without radiotherapy, n=59; claudin-low with radiotherapy, n=140; basal-like without radiotherapy, n=64; basal-like with radiotherapy, n=135). Both Kaplan–Meier and multivariate Cox regression analyses showed that *SLC20A1*^{high} patients with claudin-low breast cancer treated without radiotherapy had poorer prognoses than *SLC20A1*^{low} patients (KM: claudin-low, *p*=0.037, HR=3.39, 95%CI=1.07–10.71; basal-like: *p*=0.079) (KM: claudin-low, *p*=0.037; basal-like, *p*=0.079) (COX: claudin-low, HR=3.39, 95%CI=1.07–10.71; basal-like, HR=1.86, 95%CI 0.87–4.00) (Figure 4E and F, Table I).

Figure 3. Kaplan-Meier analyses of disease-specific survival show that *SLC20A1*^{high} patients with claudin-low and basal-like type breast cancers have poor clinical outcomes. (A-G) Kaplan-Meier analyses comparing disease-specific survival between *SLC20A1*^{high} and *SLC20A1*^{low} patients in the METABRIC dataset: (A) all, (B) normal-like, (C) luminal A, (D) luminal B, (E) HER2-enriched, (F) claudin-low, (G) basal-like subtypes. p-Values were calculated with the log-rank (Cochran-Mantel-Haenszel) test. *SLC20A1*^{high} patients are shown with a dark gray line, *SLC20A1*^{low} patients with a light gray line.

Moreover, *SLC20A1*^{high} patients with claudin-low or basal-like breast cancers treated with radiotherapy had poorer prognosis than *SLC20A1*^{low} patients treated with radiotherapy (KM: claudin-low, $p=0.039$; basal-like, $p=0.0011$) (COX: claudin-low, HR=2.12, 95%CI=1.03-4.37; basal-like, HR=2.46, 95%CI=1.37-4.42) (Figure 4G and H, Table I). Multivariate Cox regression analysis revealed that addition of chemotherapy as a covariate was also associated with a poor prognosis (No radiotherapy: claudin-low, HR=3.73, 95%CI=1.16-11.98; basal-like, HR=1.89, 95%CI=0.88-4.06) (Radiotherapy: claudin-low, HR=2.45, 95%CI=1.18-5.10; basal-like, HR=2.37, 95%CI=1.31-4.27) (Table I). These results strongly suggest that unlike chemotherapy, radiotherapy is not an effective treatment for *SLC20A1*^{high} patients with claudin-low or basal-like breast cancer.

SLC20A1 siRNA knockdown leads to suppression of MCF-7, MDA-MB 231 and MDA-MB 468 cell viability. Given the results summarized in the previous sections, we investigated the possibility that *SLC20A1* could serve as a therapeutic target for the treatment of claudin-low and basal-like breast

cancers. WST-8 cell viability assays with MCF-7 (luminal A type), MDA-MB 231 (claudin-low type) and MDA-MB 468 (basal-like type) cells showed that *SLC20A1* KD using targeted siRNA suppressed cell viability in all three lines (Figure 5A-C). This suggests *SLC20A1* contributes to cancer cell survival/proliferation in claudin-low, basal-like and ER+ breast cancer subtypes. Moreover, *SLC20A1* could be a therapeutic target as well as a prognostic marker in these cancers.

SLC20A1 siRNA knockdown leads to suppression of *in vitro* tumor-sphere formation by *ALDH1*^{high} cells. To assess the role of *SLC20A1* in *ALDH1*^{high} CSCs, we used ALDEFLUOR assays to examine the effects of *SLC20A1* depletion in *ALDH1*^{high} MDA-MB 231 and MDA-MB 468 cells on tumor-sphere formation. *SLC20A1* KD led to a decrease in *ALDH1*^{high} cell numbers and suppression of *in vitro* tumor-sphere formation by *ALDH1*^{high} MDA-MB 231 and MDA-MB 468 cells (Figure 5D-K). These results suggest that *SLC20A1* is essential for tumor formation by *ALDH1*^{high} breast CSCs in claudin-low and basal-like breast cancers.

Discussion

In this study, we revealed that *SLC20A1* is more highly expressed in breast cancer than normal tissue (Figure 1A and B) and that high *SLC20A1* expression contributes to poor prognosis in ER+ (luminal A or B), claudin-low and basal-like breast cancers (Figure 3, Table I). *SLC20A1*^{high} patients with claudin-low or basal-like breast cancers treated with chemotherapy did not have poorer clinical outcomes than *SLC20A1*^{low} patients (Figure 4C and D, Table I). However, *SLC20A1*^{high} patients treated with radiotherapy did have poorer clinical outcomes (Figure 4G and H, Table I). In addition, *SLC20A1* depletion suppressed both the viability of ER+ (luminal A), claudin-low and basal-like cancer cells and *in vitro* tumor-sphere formation by ALDH1^{high} cells (Figure 5).

SLC20A1 gene amplification was detected in only 0.32% of breast cancers (7/2173), and no other gene deep deletion, mutation or fusion was detected. A very low frequency of DNA methylation was detected at the CpG island of the *SLC20A1* promoter in breast cancer (Figure 1F). It is therefore thought that transcriptional regulation is important for the high *SLC20A1* expression seen in breast cancer, and it is anticipated that future study will reveal the transcriptional mechanism.

SLC20A1 is highly expressed in luminal B and HER2-enriched breast cancer subtypes (Figure 1C-E). Interestingly, unlike *SLC20A1*^{high} patients with luminal A/B, claudin-low, or basal-like breast cancers, those with the HER2-enriched type have good prognosis (Figure 3, Table I). Among patients with luminal B breast cancers, only about 20% are HER2+ (9). Thus, in *SLC20A1*^{high} patients with luminal B type cancers, the poor prognosis may not be associated with HER2 expression. The role of *SLC20A1* in the HER2-enriched subtype and its contribution to a good prognosis remains to be determined.

Because there are few molecular targeted drugs against claudin-low and basal-like breast cancer subtypes, the primary treatments consist of surgery, chemotherapy and radiotherapy (13, 15, 16). Although many claudin-low type tumors overlap with TNBC (12, 13), ER is also expressed in some claudin-low type (55), and 46.7% of claudin-low type patients (93/199) are not classified into TNBC. Therefore, the role of *SLC20A1* in the reclassification of claudin-low type needs to be examined. Clinical outcomes of *SLC20A1*^{high} patients treated with chemotherapy are as good as those of *SLC20A1*^{low} patients treated with chemotherapy, which is in contrast to patients treated without chemotherapy (Figure 4A-D, Table I). Thus, chemotherapy is an effective treatment for *SLC20A1*^{high} patients with claudin-low or basal-like breast cancer.

On the other hand, *SLC20A1*^{high} patients treated with radiotherapy showed poorer clinical outcomes than

Table I. Multivariate Cox proportional regression hazards model analyses of *SLC20A1*^{high} patients' prognoses in each subtype and without/with chemo- and radiotherapy in claudin-low and basal-like types.

	Hazard ratio	95% confidence interval	p-Value
DSS			
All	1.26*	1.07-1.49	0.0061
Normal-like	0.57*	0.28-1.15	0.12
Luminal A	1.69*	1.21-2.37	0.0022
Luminal B	1.65*	1.08-2.54	0.022
HER2-enriched	0.44*	0.26-0.75	0.003
Claudin-low	2.34*	1.28-4.25	0.0054
Basal-like	2.19*	1.38-3.46	<0.001
Without chemotherapy			
Claudin-low	3.73*	1.80-7.76	<0.001
Basal-like	4.97*	2.39-10.36	<0.001
With chemotherapy			
Claudin-low	0.80*	0.34-1.86	0.60
Basal-like	1.39*	0.78-2.47	0.27
Without radiotherapy			
Claudin-low	3.81**	1.84-7.92	<0.001
Basal-like	4.86**	2.32-10.18	<0.001
With radiotherapy			
Claudin-low	0.79**	0.34-1.85	0.58
Basal-like	1.33**	0.74-2.37	0.34
Without radiotherapy			
Claudin-low	3.39*	1.07-10.71	0.038
Basal-like	1.86*	0.87-4.00	0.11
With radiotherapy			
Claudin-low	2.12*	1.03-4.37	0.040
Basal-like	2.46*	1.37-4.42	0.0025
Without radiotherapy			
Claudin-low	3.73***	1.16-11.98	0.027
Basal-like	1.89***	0.88-4.06	0.10
With radiotherapy			
Claudin-low	2.45***	1.18-5.10	0.016
Basal-like	2.37***	1.31-4.27	0.0041

Hazard ratio; *adjusted by age; **adjusted by age and radiotherapy; ***adjusted by age and chemotherapy estimated using Cox proportional hazard model.

SLC20A1^{low} patients treated with radiotherapy (Figure 4E-H, Table I). Unlike chemotherapy, radiotherapy is not an effective treatment for *SLC20A1*^{high} patients with claudin-low or basal-like breast cancer. It is known that there are radioresistant and insensitive types of breast cancer (56, 57); indeed, irradiation accelerates repopulation of tumor cells (18-20). Moreover, most CSCs have radioresistance and are origin of tumor recurrence (22, 24). ALDH1 activity may contribute to radioresistance of ALDH1^{high} MDA-MB 231 and MDA-MB 468 breast cancer cells (24). Therefore, *SLC20A1* targeted therapy may overcome radioresistance/insensitivity of CSCs. However, the relationship between *SLC20A1* function and radioresistance/insensitivity remains unknown.

Figure 4. Kaplan–Meier analyses show that clinical outcomes of *SLC20A1*^{high} patients with claudin-low and basal-like breast cancers treated with chemotherapy are as good as those of *SLC20A1*^{low} patients, but outcomes of *SLC20A1*^{high} patients treated with radiotherapy are poorer. (A–H) Kaplan–Meier analyses comparing disease-specific survival between *SLC20A1*^{high} and *SLC20A1*^{low} with claudin-low or basal-like breast cancer patients in the METABRIC dataset treated with or without chemotherapy or radiotherapy: (A) claudin-low without chemotherapy, (B) basal-like without chemotherapy, (C) claudin-low with chemotherapy, (D) basal-like with chemotherapy, (E) claudin-low without radiotherapy, (F) basal-like without radiotherapy, (G) claudin-low with radiotherapy, (H) basal-like with radiotherapy.

In the treatment of breast cancer, chemotherapy and radiotherapy are usually part of a strict protocol that includes multiple cytotoxic drugs (15, 16). However, no details regarding the drugs administered, irradiation, or treatment term were available in the METABRIC dataset. At this point, therefore, there is a need to analyze the relationship between *SLC20A1* expression and prognosis, considering the details of the chemo- and radiotherapeutic protocols.

SLC20A1-deficient mice exhibit defective liver development accompanied by decreased cell proliferation and increased cell death (37). A key function of *SLC20A1* is mediating the uptake of Pi into cells (35, 36). In mouse embryonic fibroblasts, however, Pi uptake is not affected by *SLC20A1*-depletion (37), and *SLC20A1* overexpression in MC3T3-E1 osteoblastic cells does not change Pi uptake (38). In addition, the introduction of Pi-uptake defective *SLC20A1* (S128A) into *SLC20A1*-deficient cells restores cell viability (39). On the other hand, *SLC20A1* reportedly contributes to cell proliferation and migration *via* the Wnt/ β -catenin signaling in GH3 somatotroph adenoma cells (40), and

SLC20A1 depletion in HeLa or HepG2 cells impairs their proliferation and suppresses tumor growth in HeLa xenografted mice (39). *SLC20A1* depletion in HeLa cells leads to activation of p38 MAPK, but not ERK or JNK (39). It also sensitizes HeLa cells to TNF- α -induced apoptosis *via* JNK signaling (58). It thus appears that *SLC20A1* is involved in the regulation of Pi uptake-dependent and -independent cell proliferation and death, though the mechanism remains to be determined.

Using information-theoretical analysis, we previously identified *SLC20A1* expression as a poor prognosis marker in breast cancer (41). Based on the results of the genomic dataset analyses and *in vitro* experiments performed in the present study, we suggest that *SLC20A1* could serve as a therapeutic target as well as a prognostic marker in ER+, claudin-low and basal-like breast cancers. Moreover, our findings reveal these types of analyses to be powerful methods with which to identify molecules with the potential to effectively serve as prognosis markers and/or molecular targets for the treatment of other forms of cancer.

Figure 5. SLC20A1 knockdown using targeted siRNA suppresses the viability of MCF-7, MDA-MB 231, MDA-MB 468 cells and in vitro tumor-sphere formation by ALDH1^{high} cells. (A-C) WST-8 assays assessing MCF-7 (A), MDA-MB 231 (B), MDA-MB 468 (C) cell viability 48 h after transfection of SLC20A1-targeted siRNA. Values for the test groups are expressed relative to cells transfected with control siRNA. Bars represent means $\pm$ SE (three independent experiments). ** p <0.01, * p <0.05, Student's t -test. (D-E) Numbers of ALDH1^{high} (D) MDA-MB 231 and (E) MDA-MB 468 cells. * p <0.05, ** p <0.01, Student's t -test. Bars represent means $\pm$ SD (three independent experiments). (F-G) In vitro tumor-sphere formation by ALDH1^{high}, MDA-MB 231 and MDA-MB 468 cells (three independent experiments). Representative images are shown (F-G), scale bar, 50 μ m; (H-I) areas of tumor-spheres, * p <0.05, Student's t -test, bars represent means $\pm$ SE; and (J-K) numbers of tumor-spheres, N.S.: Not significant, * p <0.05, Student's t -test, bars represent means $\pm$ SD.

Conflicts of Interest

The Authors declare that they have no competing interests in relation to this study.

Authors' Contributions

C.O., S.T., H.M., A.O., C.M. and Y.H. performed the experiments; C.O., S.T., H.M., A.O., C.M., I.M., and T.F. performed bioinformatics; Y.N., Y.H., Y.K., Y.H., T.S., Y.M., and K.S. supplied experimental materials and resources; C.O., S.T. and K.A. conceived the study; C.O., S.T. and K.A. drafted the manuscript; C.O., S.T., H.M., A.O., Y.H., Y.N., T.S., Y.M., K.S. and K.A. contributed to discussion and review of the final manuscript; all the authors approved the final manuscript.

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