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Diversity of ER-positive and HER2-negative breast cancer stem cells attained using selective culture techniques

Satoshi Sueoka[1,2], Azusa Kai[1], Yukino Kobayashi[3], Masaoki Ito[4], Shinsuke Sasada[1], Akiko Emi[5], Noriko Gotoh[6], Koji Arihiro[7], Koh Nakayama[3], Morihito Okada[1] & Takayuki Kadoya[1,2]✉

Breast cancer stem cells are a promising therapeutic target in cancer. We explored breast cancer stem cell diversity and establish a methodology for selectively culturing breast cancer stem cells. We collected breast cancer tissues from surgical samples of treatment-naïve patients with estrogen receptor (ER)-positive, human epidermal growth factor receptor 2 (HER2)-negative breast cancer. Following isolation, cells were subjected to spheroid culture on non-adherent plates. Of the 57 cases, successful culture was achieved in 48 cases, among which the average ratio of CD44+/CD24− breast cancer cells increased from 13.8% in primary tumors to 61.6% in spheroids. A modest number of spheroid cells successfully engrafted in mice and subsequently re-differentiated within the murine environment, confirming their stemness. ER expression in spheroid cells exhibited negative conversion in 52.1% of cases. The proportion of Twist-, Snail-, and Vimentin-positive cells increased from 43.8%, 12.9%, and 7.7–75.0%, 58.1%, and 37.7%, respectively. ER-positive, HER2-negative breast cancer stem cells were classified into two groups using DNA microarrays. Gene Ontology analysis unveiled higher expression of immune response-related genes in one group and protein binding-associated genes in the other. We demonstrated stable and selective culture of breast cancer stem cells from patient-derived breast cancer tissue using spheroid cultures.

Keywords Breast cancer, Stem cell, Epithelial–mesenchymal transition, CD44, CD24, Spheroid culture

Abbreviations

EMT	Epithelial–mesenchymal transition
ER	Estrogen receptor
HER2	Human epidermal growth factor receptor 2
NSG	NOD scid gamma
pCR	Pathological complete response
PDSX	Patient-derived stem cell xenograft
PgR	Progesterone receptor

Cancer stem cells, capable of self-renewal and pluripotency, are considered to be involved in cancer development and differentiation. Even in small numbers, cancer stem cells possess the capacity to form metastatic lesions[1]. They display resistance to anticancer drugs and radiation therapy, thereby increasing the likelihood of their survival after treatment and their involvement in cancer recurrence and metastasis[2,3]. Consequently, cancer stem cells are the ultimate curative target in cancer.

[1]Department of Surgical Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan. [2]Department of Breast Center, Shimane University Hospital, Izumo, Japan. [3]Department of Pharmacology, Asahikawa Medical University, Asahikawa, Japan. [4]Department of Surgery, Kindai University Hospital, Osakasayama, Japan. [5]Department of Breast Surgery, Hiroshima City North Medical Center Asa Citizens Hospital, Hiroshima, Japan. [6]Division of Cancer Cell Biology, Cancer Research Institute, Kanazawa University, Kanazawa, Japan. [7]Department of Anatomical Pathology, Hiroshima University Hospital, Hiroshima, Japan. ✉email: takayukikadoya@gmail.com

It is possible to selectively culture cancer stem cells from a patient's breast cancer tissue. Spheroid culture is one of the most promising methods for this purpose. However, there are various methods of spheroid culture, and the characteristics of the spheroids obtained using each method differ[4]. Scaffold-based methods and round bottom methods form spheroids in a short period owing to cell aggregation, but the cells obtained using these methods lack stem cell characteristics. In contrast, spheroid culture using non-adherent plates enables selective culture of breast cancer stem cells. In this method, breast cancer stem cells withstand anoikis in culture and expand under anchorage-independent conditions. They can grow clonally even under harsh conditions on low-cell density and low-adhesion plates[5,6]. Hinohara et al. have reported that spheroids obtained using low-cell density and low-adhesion plate culture are not formed through simple cell aggregation; rather, they are formed through clonal expansion of single stem cells[7].

In the field of breast cancer, to our knowledge, selective culture of breast cancer stem cells from a large number of patient tissues has not been reported. Therefore, the biological characteristics and gene expression of breast cancer stem cells remain unresolved. In this study, we demonstrate selective and stable culture of breast cancer stem cells from patient-derived breast cancer tissue using a long-term spheroid culture method with low-cell density and low-adhesion plates. The biological characteristics and gene expression diversity of breast cancer stem cells were explored. Typically, breast cancer stem cells are characterized by their surface expression of CD44 and CD24[8,9]. However, these cells exhibit further diversity[10–14]; other surface markers, such as CD133, CD49f, and CD61, have also been reported[10–13]. Thus, specific markers for these cells have not yet been identified. Therefore, in the present study, the spheroids selected and cultured from clinical specimens were immunostained for CD44/CD24, the most widely used stem cell markers. In addition, stemness was verified by demonstrating the proliferative potential through transplantation into mice and differentiation potential within the mouse body. To the best of our knowledge, this is the largest study of selective culture of breast cancer stem cells from patient-derived breast cancer tissues. Furthermore, this is the first study to show the dedifferentiation and differentiation of breast cancer stem cells derived from patient tissues over time using a mouse metastatic model.

Results

Spheroid culture from patient-derived breast cancer tissues

Table 1 outlines the clinical attributes of the 48 patients with breast cancer, whose surgical specimens were successfully used for selective culture of breast cancer stem cells. All patients were ER-positive and HER2-negative, with tumor diameters ranging from T1 to –T3; notably, 17 cases (35.4%) exhibited positive lymph node metastases. Spheroid images on days 13 and 27 of the culture, accompanied by a growth curve depicting the spheroid count per plate, are shown in Fig. 1a. The number of spheroids continued to increase until day 28, beyond which there was a marginal increase. Most cells comprising the spheroids were GATA3-positive breast

Characteristic	Number of patients ($n=48$)
Age (years)	
Median (range)	63 (39–91)
Histological type	
Ductal	40 (83.3%)
Lobular	3 (6.3%)
Mucinous	5 (10.4%)
Tumor size	
T1c (> 1 cm, ≤ 2 cm)	14 (29.2%)
T2 (> 2 cm, ≤ 5 cm)	28 (58.3%)
T3 (> 5 cm)	6 (12.5%)
Node metastasis	
Positive	17 (35.4%)
Nuclear grade	
1	10 (20.8%)
2	11 (22.9%)
3	27 (56.3%)
Ki67 labeling index	
< 20%	16 (33.3%)
≥ 20%	32 (66.7%)
ER (Allred score)	
Median (range)	7 (6–8)
PgR (Allred score)	
Median (range)	7 (0–8)

Table 1. Clinical characteristics of the 48 patients with breast cancer. *ER* estrogen receptor, *PgR* progesterone receptor.

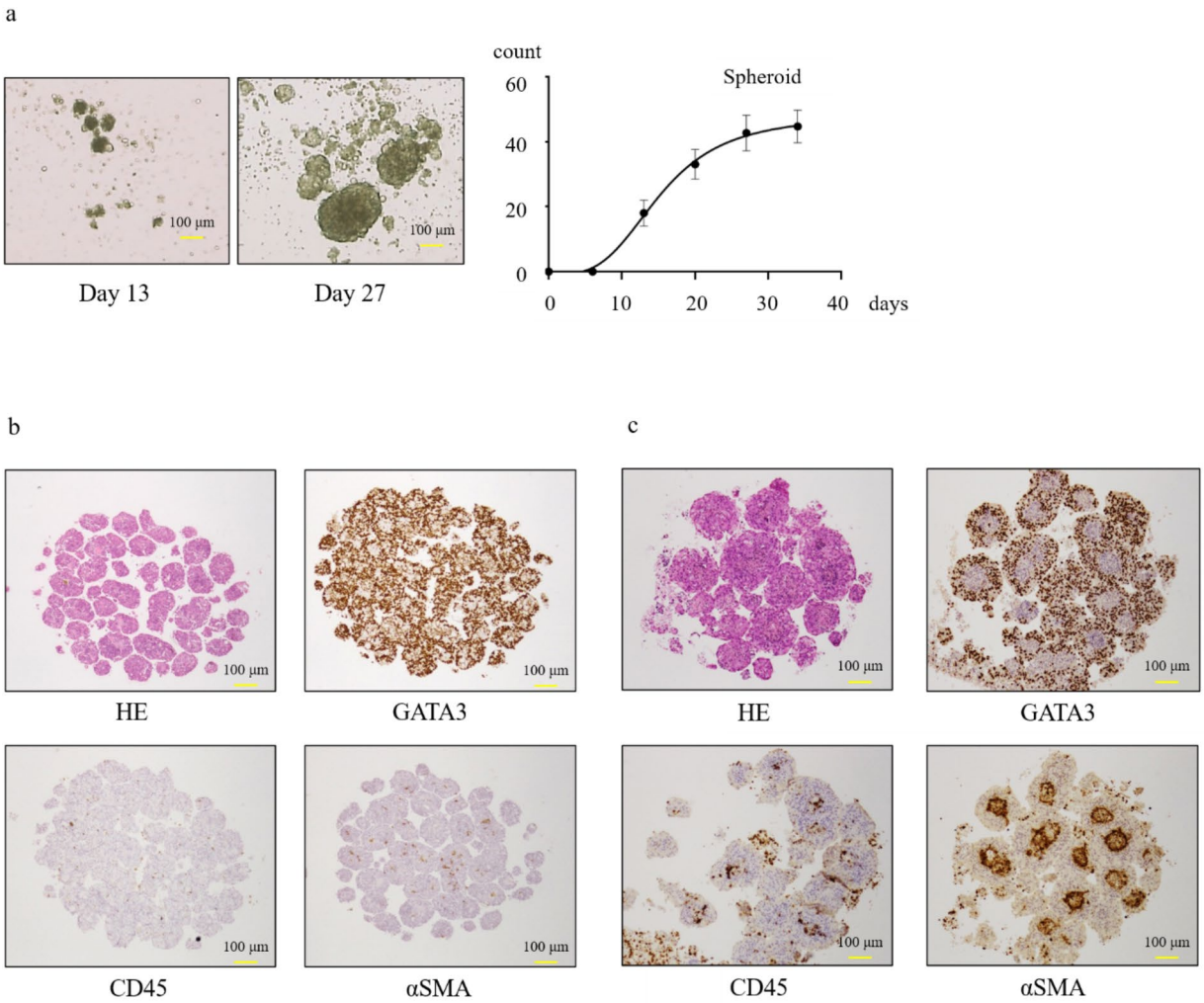

Fig. 1. Cell proliferation by spheroid culture and spheroids with cells. (**a**) Spheroids on days 13 and 27 and changes in the number of spheroids per plate. (**b**,**c**) Representative spheroid immunostained specimens for HE, GATA3, CD45, and αSMA. Magnification: 40×; Scale bar: 100 µm.

cancer cells; they also encompassed CD45 + leukocytes and αSMA + fibroblasts (Fig. 1b). The proportions of each cell type demonstrated variations across cases, with some cases exhibiting relatively high counts of leukocytes and fibroblasts (Fig. 1c).

Increase in the stemness of breast cancer stem cells with spheroid culture

The stemness of cells procured through spheroid culture was evaluated using CD44 and CD24 immunostaining. A comparative analysis between the primary tumor tissues and spheroids revealed a considerable increase in the CD44+/CD24− ratio within the spheroids, averaging at 61.6% versus 13.8%, respectively, in the primary tumors (Fig. 2a). We examined the changes in CD44+/CD24− ratios per case; 46 cases (95.8%) exhibited an increase in spheroids relative to that in the primary tumors, whereas 2 cases (4.2%) showed no change (Fig. 2b).

Alterations in hormone receptor positivity in breast cancer stem cells

The number of cases demonstrating strong ER positivity reduced to 12 (25.0%) in the spheroid culture, whereas all cases displayed strong ER positivity (Allred score of 6–8) in the primary tumors. Furthermore, ER expression in 25 cases (52.1%) changed to negative (Allred score 0–2), and 3 cases (6.3%) showed no changes. Additionally, none of the cases showed an increase in ER expression (Fig. 2c). For progesterone receptor (PgR) expression, the number of cases of strong PgR positivity (Allred score 6–8) dropped from 31 cases (64.6%) to 8 cases (16.7%) in spheroid culture. Additionally, 10 cases (20.8%) showed no changes, whereas 2 cases (4.2%) showed increased PgR expression (Fig. 2d). A downward trend was observable after spheroid culture, although the change in the Allred score for PgR was less pronounced than that for ER.

Changes in mesenchymal marker positivity in breast cancer stem cells

Notable shifts were observed in mesenchymal marker positivity rates before and after spheroid culture. Twist1 expression increased from 43.8% in the primary tumors to 75.0% in the spheroids, whereas Snail expression

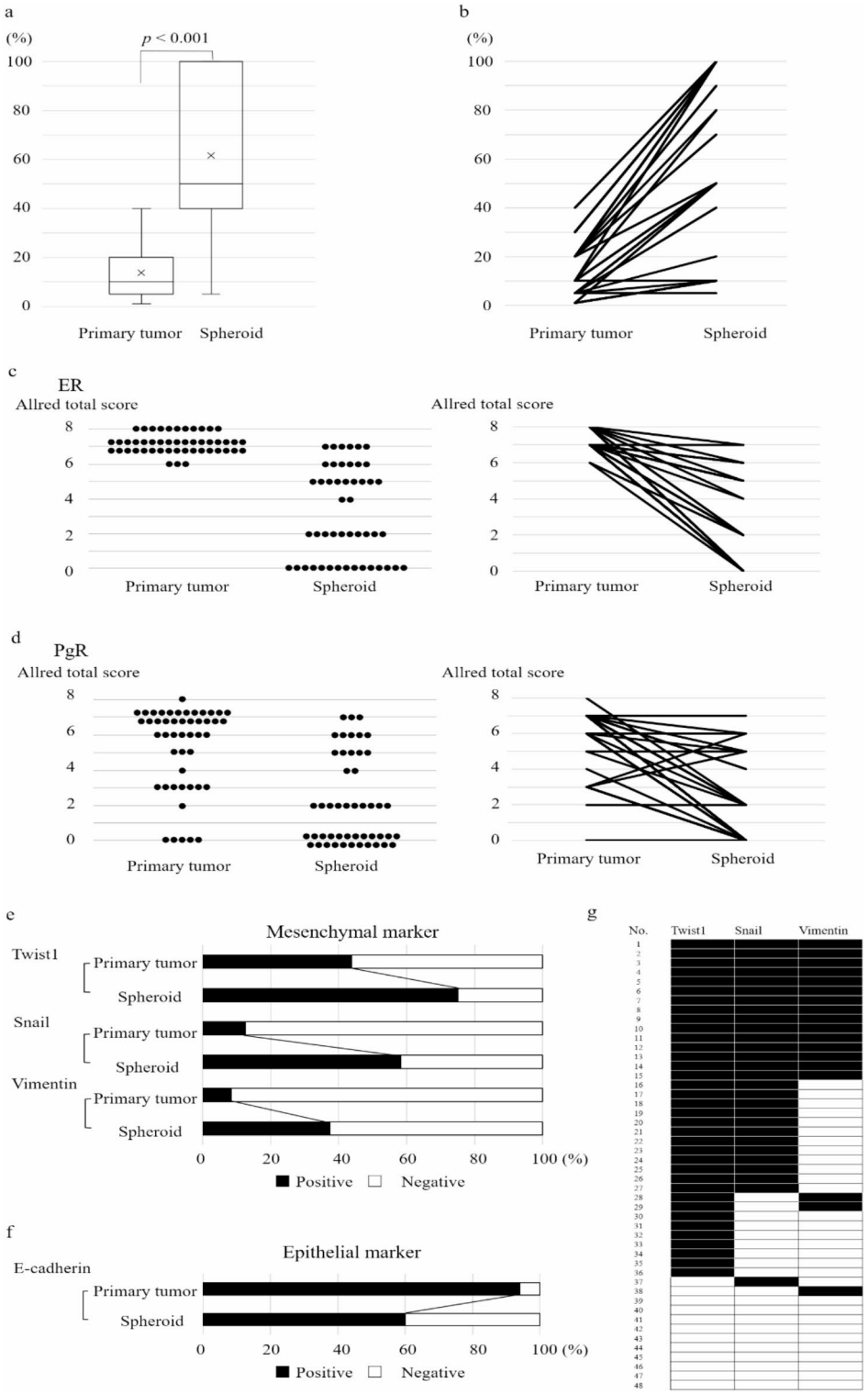

Fig. 2. Comparison of the pathological findings of primary tumors and spheroids. (**a**,**b**) Changes in the proportion of CD44 + and CD24− cells observed via spheroid culture. (**c**,**d**) Changes in ER and PgR expression observed with spheroid culture. (**e**,**f**) Changes in the expression of mesenchymal markers and epithelial markers in primary tumors and spheroids, black shading: positive; white: negative. Mesenchymal marker expression increased and epithelial marker expression decreased in spheroids. (**g**) Mesenchymal marker expression in each case. The expression of mesenchymal markers in each case is divided into four patterns. *ER* estrogen receptor, *PgR* progesterone receptor. Results with a *p*-value of < 0.05 were considered statistically significant.

increased from 12.9 to 58.1%, and Vimentin expression increased from 7.7 to 37.7% (Fig. 2e). Conversely, the expression of the epithelial marker E-cadherin decreased from 95.4% in the primary tumors to 59.8% in the spheroids (Fig. 2f). The expression of mesenchymal markers for each case revealed four delineated patterns (Twist1+/Snail+/Vimentin+, Twist1+/Snail+/Vimentin−, Twist1+/Snail−/Vimentin−, and Twist1−/Snail−/Vimentin−), highlighting variations in the mesenchymal properties of breast cancer stem cells even for the same case of ER-positive and HER2-negative breast cancer (Fig. 2g).

Differentiation potential of breast cancer stem cells

Eleven breast cancer stem cell samples, selected and cultured via the spheroid culture method, were transplanted into the subcutaneous tissue of NOD scid gamma (NSG) mice, and patient-derived stem cell xenografts (PDSXs) were successfully established in five cases (45.5%). However, a clear correlation was not observed between the clinical and pathological features of the primary tumors and spheroids and the success of PDSX establishment (Table 2). Among the five cases in which PDSXs were successfully established, a comparison of CD44+/CD24− ratios among the primary tumors, spheroids, and PDSXs revealed that the increased CD44+/CD24− ratio observed after spheroid culture decreased to a level similar to that of the primary tumors in the PDSXs (Fig. 3a). Additionally, the ratio of hormone receptor-positive cells decreased in the spheroids compared with that in the primary lesion and increased again in PDSXs. However, both spheroids and PDSXs had a single case of negative conversion of the hormone receptor (Fig. 3b, case 2). Conversely, the Snail (mesenchymal marker)-positive ratio increased in spheroids compared with that in primary tumors and then decreased in PDSXs (Fig. 3c).

Proliferative potential of breast cancer stem cells

Both primary tumor and breast cancer stem cells obtained through spheroid culture were transplanted into mice for a comparative evaluation of their proliferative potential. To ensure sufficient cell numbers, tumors were collected from 5 additional cases, in addition to the 57 cases used for pathological examination. Different cell numbers were set, and the cells were transplanted into the following four mammary gland sites: the left and right thoracic and abdominal regions. Transplantation began with 2.5×10^4 cells in both groups, and upon the transplantation of 2.5×10^6 cells, spheroid-cultured cells successfully engrafted. Ultimately, tumor formation occurred in 3 out of 10 instances where the spheroid-cultured cells were used; however, the same number of cells without spheroid culture did not successfully engraft (Table 3).

Genetic diversity of breast cancer stem cells

DNA microarray analysis was performed on breast cancer stem cells selected and cultured from 14 surgical specimens. Clustering analysis based on gene expression levels revealed two distinct groups of breast cancer stem cells (Fig. 4a). This classification remained consistent regardless of cutoff value adjustments (Supplementary Fig. S1). Gene Ontology analysis of these groups unveiled gene groups with substantially different gene expression levels (Fig. 4b). One group showed increased expression of immune response-related genes, such as *TNFRSF17*, *POU2AF1*, and *CD79A* (immune response group) (Table 4), whereas the other group exhibited relatively high expression of genes involved in protein binding, including *SELENBP1* and *ICA1* (protein interaction response group) (Table 5).

Discussion

To the best of our knowledge, this is the largest study to selectively culture breast cancer stem cells from patient-derived breast cancer tissue. Furthermore, this is the first study to demonstrate the dedifferentiation and differentiation of breast cancer stem cells derived from patient-derived tissues over time.

The spheroid culture system is thought to simulate cellular dynamics in the body more effectively than the 2D culture system[15–17]. It is also used to enrich cells with stem cell potential in cancers of the breast[18,19], brain[20], and head and neck[21]. Different spheroid culture methods exist, and the characteristics of the resulting cells vary considerably. Most previous studies on breast cancer spheroids have focused on the 3D structure of spheroids using aggregation rather than stem cell selectivity. Moreover, there have been reports on drug sensitivity when using patient-derived spheroids[22]. However, even in the same spheroid culture, if the seeding density is high, non-stem cells can aggregate to form spheroids, thereby reducing the efficiency of selectively culturing cancer stem cells[7]. To improve the efficiency of cancer stem cell selection to the greatest extent possible, we performed spheroid selection by seeding at a low cell density on low-adhesion flat plates. To our knowledge, no study has selectively cultured breast cancer stem cells from breast cancer tissues of a large number of patients and examined their biological characteristics. In order to selectively culture breast cancer stem cells from breast cancer tissue and examine their biological characteristics, we developed a spheroid culture method that enables clonal expansion of breast cancer stem cells using low-adhesion plates. We collected specimens from 57 cases and successfully established spheroid culture in 48 of these cases, achieving a success rate of 84.2%. In the 9 cases wherein culture was unsuccessful, cell isolation was challenging, probably due to the tumor type being mucinous carcinoma. Additionally, although the exact mechanism remains unclear, tumor-specific factors may have also contributed.

We observed a substantial increase in the CD44+/CD24− ratio in cells obtained via spheroid culture compared with that of primary breast cancer cells. This marker is the most widely used for breast cancer stem cells and provides evidence that the selectively cultured cells are indeed breast cancer stem cells. Furthermore, the transplantation of spheroid-cultured cells into mice and creation of the PDSX model demonstrated the pluripotent and proliferative nature of these stem cells. Regarding pluripotency, selectively cultured breast cancer stem cells were shown to differentiate into distinct cell types, as evidenced by increased expression of hormone receptors and epithelial markers. Regarding proliferation capacity, these selectively cultured breast cancer stem cells exhibited a tendency to form tumors even when only a small number of cells were transplanted.

Case	Number of transplanted spheroid cells	PDSX	Primary tumor							Spheroid				
			Histological type	Tumor size	Node metastasis	Nuclear grade	Ki67 labeling index (%)	ER (Allred score)	PgR (Allred score)	ER[2] (Allred score)	PgR[3](Allred score)	Twist1 (%)	Snail (%)	Vimentin (%)
1	8.5×10^4	×	Ductal	T2	Negative	1	6	7	7	4	4	70	50	50
2	2.4×10^6	×	Ductal	T3	Positive	3	60	7	4	5	5	60	30	30
3	5.5×10^5	×	Ductal	T1c	Negative	2	15	7	2	6	2	70	50	20
4	2.2×10^5	○	Ductal	T1c	Negative	3	40	7	7	7	2	40	40	30
5	1.8×10^6	○	Ductal	T2	Negative	1	5	8	7	2	7	70	60	20
6	5.4×10^4	○	Ductal	T2	Negative	1	20	8	7	4	2	50	20	10
7	2.6×10^5	×	Ductal	T2	Negative	2	3	8	6	2	0	40	20	10
8	1.5×10^6	×	Ductal	T2	Positive	3	95	8	5	4	5	70	40	30
9	5.8×10^5	○	Ductal	T2	Positive	3	27	7	7	4	0	80	50	40
10	4.5×10^5	×	Ductal	T2	Negative	3	49	7	3	2	3	50	40	20
11	1.1×10^6	○	Ductal	T2	Negative	3	87	7	7	0	0	80	60	50

Table 2. Details regarding breast cancer stem cell transplantation cases in mice. *PDSX* patient-derived stem cell xenograft, *ER* estrogen receptor, *PgR* progesterone receptor.

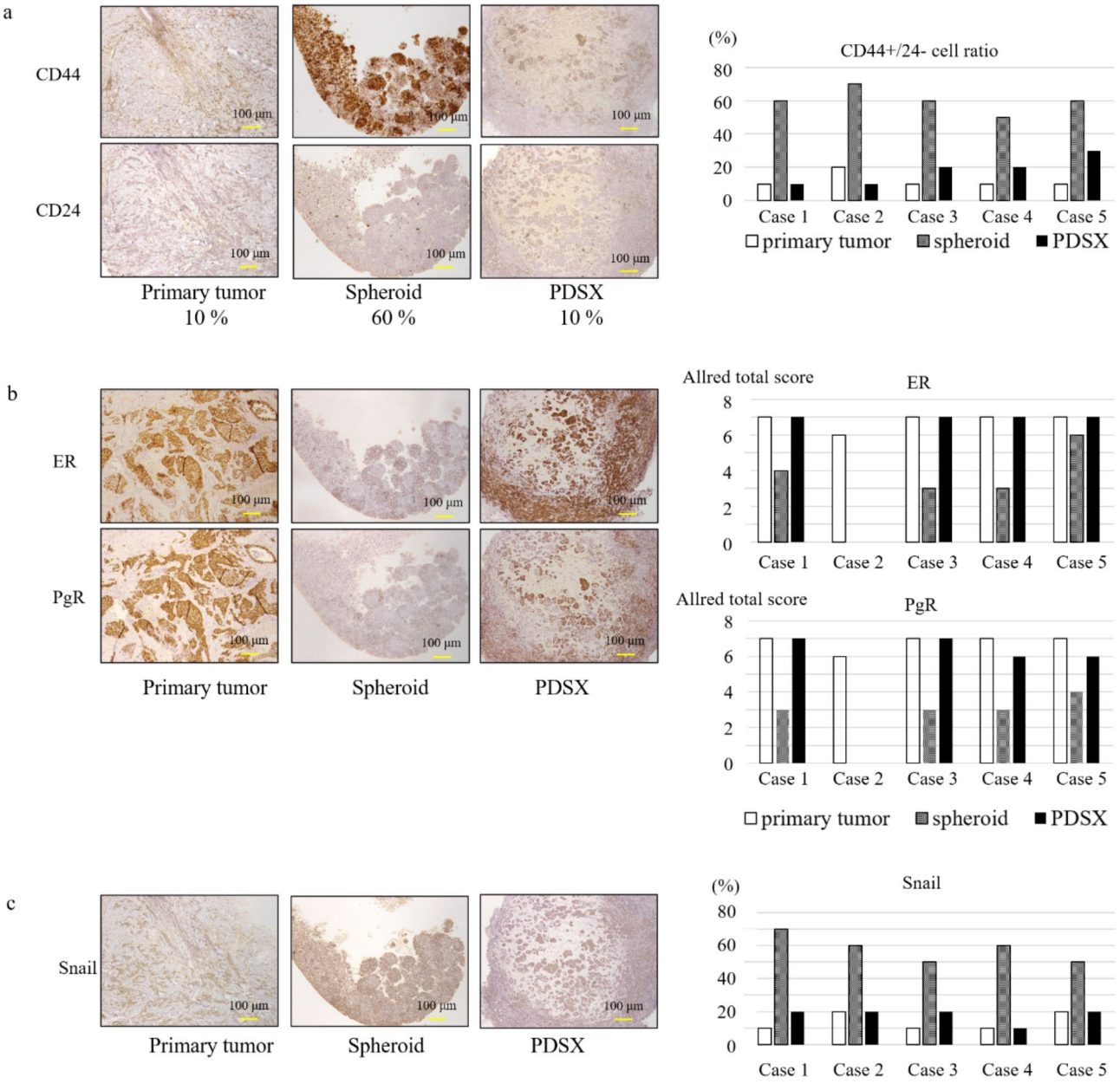

Fig. 3. Comparison of the pathological findings of primary tumors, spheroids, and PDSXs. (**a**) Changes in the percentage of CD44 + and CD24− cells. (**b**) Changes in the expression of ER and PgR. (**c**) Changes in the expression of mesenchymal marker (Snail). White: primary tumor; black dots: spheroids; black: PDSX. *ER* estrogen receptor, *PgR* progesterone receptor, *PDSX* patient-derived stem cell xenografts. Magnification: 40×; Scale bar: 100 μm.

However, due to the limited cell numbers and the inherent variability of clinical samples, it was not possible to obtain statistically significant data. For example, Ki-67, a common metric for assessing proliferative activity in breast cancer, was influenced by variability in Ki-67 values among our clinical samples, which prevented us from fully accounting for individual differences. Despite these limitations, the observed tumor-forming tendency aligns with previous reports that CD44+/CD24− populations exhibit tumorigenic potential[8]. Our findings further revealed decreased/negative expression of ER and PgR in breast cancer stem cells compared with their expression in primary tumors. Additionally, many cases demonstrated positive Twist1, Snail, and Vimentin expression. These results clinically support epithelial–mesenchymal transition (EMT) of breast cancer stem cells, which causes cancer metastasis[23]. Moreover, here, DNA microarray analysis demonstrated that ER-positive, HER2-negative breast cancer can be classified into two groups based on their gene expression profiles. Subsequently, Gene Ontology analysis revealed differential gene expression sets between the groups, labeled as the "immune response group" and "protein interaction response group."

Case	Number of transplanted cells	Before selective culture of stem cells	After selective culture of stem cells
A	1.0×10^5	–	–
	5.0×10^5	–	–
B	2.5×10^4	–	–
	1.0×10^6	–	–
C	2.5×10^4	–	–
	2.5×10^6	–	+
D	2.5×10^4	–	+
	2.5×10^6	–	+
E	2.5×10^4	–	–
	2.5×10^6	–	–

Table 3. Tumor formation ability of patient-derived cells before and after selective spheroid culture in mice.

Our study revealed several key findings. First, we successfully established a method to selectively culture breast cancer stem cells from patient-derived breast cancer tissues, achieving a notably high success rate (84.2%; 48/57 cases). Second, we confirmed a reduction in hormone receptor expression and the acquisition of mesenchymal attributes in breast cancer stem cells, along with their epithelial differentiation in metastatic organs employing stem cells derived from clinical samples. Finally, breast cancer stem cells (even within the same clinical subtype) demonstrated biological and genetic diversity. Consequently, customizing pharmacotherapy approaches for different groups of breast cancer stem cells becomes feasible, bringing us closer to the development of curative treatments. Moreover, the coexistence of diverse cells (such as lymphocytes and fibroblasts) within spheroids indicates the formation of a tumor microenvironment. Cancer stem cells survive harsh conditions during metastasis and engraftment using the cellular environment surrounding them[24–27]. Insights into spheroids may lead to elucidation of the cancer microenvironment.

The study has certain limitations. First, we solely focused on ER-positive and HER2-negative breast cancer cases and excluded the analysis of other clinical subtypes, such as triple-negative and HER2-positive breast cancers. These subtypes are typically subjected to neoadjuvant chemotherapy, and there are challenges in acquiring sufficient tumor tissue samples. Second, the relatively low recovery rate of breast cancer stem cells poses challenges for simultaneously conducting multiple analyses, including immunostaining for ER, PgR, CD44, and CD24; genetic analysis; and mouse transplantation, within the same case. Efficient culture techniques should be developed in the future.

Despite these limitations, our results are considered applicable to the realization of stem cell-targeted personalized medicine and prognostic predictions. The effects of anticancer drugs and prediction of metastasis and recurrence are particularly determined based on cancer stem cells. Currently, companion diagnostics for treatment selection and immunostaining for prognostic prediction are being conducted. However, these tests target the entire population of cancer cells, including non-stem cells. Our study serves as a foundation for achieving selection of treatments targeting breast cancer stem cells and for enhancing the accuracy of prognostic prediction by targeting these cells.

In conclusion, our study demonstrates the selective and stable culture of breast cancer stem cells from breast cancer tissues through spheroid culture. Our results also reveal the complex biological and genetic diversity among breast cancer stem cells in ER-positive and HER2-negative breast cancer cases. Understanding the differentiation mechanism of cells may facilitate the modulation of differentiation and dedifferentiation in breast cancer cells. This could enhance the effectiveness of pharmacotherapy by regulating differentiation, ultimately yielding innovative strategies for manipulating breast cancer stem cell differentiation.

Methods

Patients

The selection criteria for this study were patients with ER-positive and HER2-negative breast cancer who had not received neoadjuvant pharmacotherapy and were diagnosed with invasive carcinoma with a tumor size of ≥2 cm based on imaging conducted between March 2020 and January 2022. This study was conducted in accordance with the ethical principles set by the Institutional Review Board and the Declaration of Helsinki (1964), following approval from the Ethics Committee of Hiroshima University (E-1658, June 24, 2019). All participants provided informed consent after being duly briefed about the utilization of a portion of their surgical specimen for experimental purposes.

Cell culture

Thin sections, measuring 20 mm in diameter and 1 mm in thickness, were procured from the tumor site of 48 patients immediately after surgery and subsequently minced. Cell separation was achieved through enzymatic treatment involving collagenase (Sigma-Aldrich, St. Louis, MO, USA) and DNase (Sigma-Aldrich); the minced tissue was incubated with the enzymes at 37 °C for 1 h with stirring. After passing through a cell strainer, the cells were cultured in a non-adhesive plate (Corning, Corning, NY, USA) at 37 °C under 5% CO_2 for 5 weeks, with Dulbecco's modified Eagle's medium/F12 (1:1) (Thermo Fisher Scientific, Waltham, MA, USA). This medium was supplemented with 2% B-27 (Thermo Fisher Scientific), basic fibroblast growth factor (2 ng/mL; Wako, Osaka, Japan), epidermal growth factor (2 ng/mL; Sigma-Aldrich), penicillin G (100 U/mL), and streptomycin

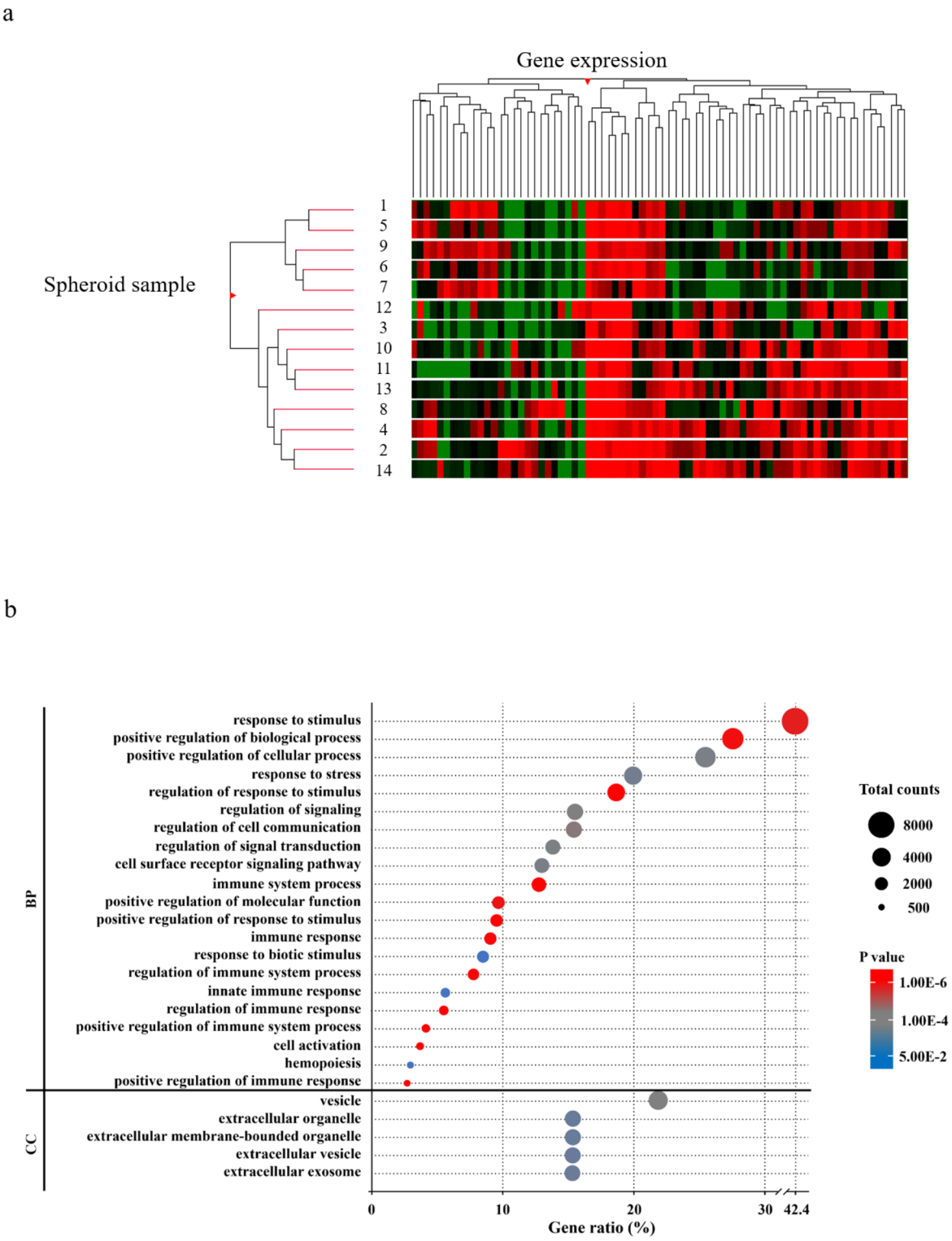

Fig. 4. Gene analysis findings. (**a**) Classifying breast cancer stem cells using clustering analysis. Breast cancer stem cells were broadly classified into two groups. (**b**) Gene Ontology enrichment analysis between the groups. Larger circles represent higher counts. *GO* Gene Ontology, *BP* biological process, *CC* cellular component. Results with a *p*-value of < 0.05 were considered statistically significant.

sulfate (0.1 mg/mL; Wako). Regarding the cell seeding density, it has been reported that under low-density conditions, such as 5,000 cells/mL, mammospheres form through the clonal expansion of single cells rather than simple cell aggregation[7]. Therefore, in this study, we also established the cell seeding density at 5,000 cells/mL as a standard. Cell aggregates with a diameter of ≥ 100 μm were defined as spheroids.

Gene symbol	*p* ([immune response group] vs. [protein interaction response group])	GO molecular function term
TNFRSF17	0.0013	Adaptive immune response//lymphocyte homeostasis//signal transduction
IRF4	0.0013	Transcription from RNA polymerase II promoter//peptidyl-lysine methylation//cytokine-mediated signaling pathway
STAP1	0.0013	Transmembrane receptor protein tyrosine kinase signaling pathway//positive regulation of gene expression//negative regulation of macrophage chemotaxis
POU2AF1	0.0013	Regulation of transcription, DNA-templated//transcription from RNA polymerase II promoter//humoral immune response
CD79A	0.0018	Adaptive immune response//B cell differentiation//B cell proliferation//B cell activation
ZNF215	0.0018	RNA polymerase II transcription factor activity, sequence-specific DNA binding//transcription factor activity, sequence-specific DNA binding//transcription factor
TAF4B	0.0023	DNA binding//transcription factor activity, sequence-specific DNA binding//protein heterodimerization activity
CD180	0.0031	B cell proliferation involved in immune response//inflammatory response//innate immune response
IKZF3	0.0035	RNA polymerase II regulatory region sequence-specific DNA binding//transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific binding//transcription factor activity, sequence-specific DNA binding
PIK3CG	0.0039	Protein kinase activity//protein serine//threonine kinase activity//protein binding

Table 4. Genes with high expression in the immune response group. *GO* Gene Ontology.

Gene symbol	*p* ([protein interaction response group] vs. [immune response group])	GO molecular function term
TRPS1	0.0015	Negative regulation of transcription from RNA polymerase II promoter//skeletal system development//chondrocyte differentiation
SELENBP1	0.0023	Protein binding//selenium binding//selenium binding
TC2N	0.0023	Calcium ion binding//calcium-dependent phospholipid binding//syntaxin binding
TSPAN15	0.0031	Plasma membrane//integral component of plasma membrane//cell surface
MGST2	0.0031	Nuclear envelope//endoplasmic reticulum membrane//plasma membrane
ICA1	0.0048	Protein binding//protein domain specific binding//molecular function
FOXA1	0.0059	Transcription factor binding//protein domain specific binding//sequence-specific DNA binding
GATA3	0.0062	Protein binding//transcription factor binding//zinc ion binding
PPP2R4	0.0062	Peptidyl-prolyl cis-trans isomerase activity//receptor binding//protein binding
PROM2	0.0066	Cholesterol binding

Table 5. Genes with high expression in the protein interaction response group. *GO* Gene Ontology.

Immunohistochemistry

Immunostaining was conducted on cultured spheroids and primary breast cancer lesions using an array of antibodies, including ER (anti-human rabbit monoclonal, clone SP1, dilution 1/1; Roche, Basel, Switzerland), PgR (anti-human rabbit monoclonal, clone IE2, dilution 1/1; Roche), HER2 (anti-human rabbit polyclonal, clone SK001, dilution 1/1; Dako, Santa Clara, CA, USA), CD24 (anti-human mouse monoclonal, clone SN3b, dilution 1/50; Thermo Fisher Scientific), CD44 (anti-human rabbit monoclonal, clone SP37, dilution 1/1; Roche), CD45 (anti-human mouse monoclonal, clone 2B11 + PD7/26, dilution 1/1; Dako), CD19 (anti-human mouse monoclonal, clone BT51E, dilution 1/50; Leica Biosystems, Nussloch, Germany), GATA3 (anti-human mouse monoclonal, clone L50-823, dilution 1/1; Roche), αSMA (anti-human mouse monoclonal, clone 1A4, dilution 1/1; Dako), Twist1 (anti-human rabbit polyclonal, clone GTX50821, dilution 1/100; Gene Tex, Irvine, CA, USA), Snail (anti-human goat polyclonal, clone ab53519, dilution 1/300; Abcam, Cambridge, UK), Vimentin (anti-human mouse monoclonal, clone V9, dilution 1/1; Dako), and E-cadherin (anti-human mouse monoclonal, clone 36, dilution 1/1; Roche). We assessed ER, PgR, and HER2 expression following the protocols outlined by the American Society of Clinical Oncology/College of American pathologists[28,29]. The slides were examined simultaneously using a multi-observer microscope by two investigators blinded to the corresponding clinicopathological data.

Patient-derived stem cell xenografts

The transplantation of breast cancer stem cells was performed in 8–9-week-old NSG mice (Charles River Laboratories Japan, Yokohama, Japan). Cells from each case were transplanted into a single mouse, with a total of 16 mice used in the experiment ($n = 11$ in Table 2 and $n = 5$ in Table 3). Breast cancer stem cells were combined with 100 µL Matrigel and subcutaneously transplanted in proximity to the mammary gland. Upon attaining a size of 10 mm, as measured from the body surface, the formed tumors were excised and subjected to pathological evaluation. All experimental procedures involving mice were carried out in accordance with relevant guidelines and regulations and in compliance with ARRIVE guidelines (https://arriveguidelines.org). Ethical approval was obtained from the Hiroshima University Animal Ethics Committee (approval ID: A20-151, January 5, 2021).

DNA microarray and Gene Ontology analysis

A GeneChip ClariomS Human Array system (Affymetrix; Thermo Fisher Scientific) was used to explore differences in gene expression in breast cancer stem cells. Microarray experimentation and data analysis were performed by Kurabo Industries (Osaka, Japan). RNA from spheroids was extracted using the GeneChip WT PLUS Reagent kit (Affymetrix; Thermo Fisher Scientific) according to the manufacturer's instructions; 100 ng of total RNA was used for microarray analysis. The resultant single-stranded cRNA was fragmented, biotin-labeled, and subjected to hybridization using the Clariom S Human Array. Following the manufacturer's protocol, the array was washed, stained, and scanned using the Affymetrix 450 Fluidics Station and GeneChip Scanner 3000 7G (Affymetrix; Thermo Fisher Scientific). Subsequent normalization and calculation of expression levels were executed using the SST-RMA method with Expression Console version 1.3 (Affymetrix; Thermo Fisher Scientific). Genes exhibiting expression shifts of 1,000-fold or greater were selected as differentially expressed genes and consequently used in generating a heatmap through hierarchical clustering. The gene expression data of each sample were analyzed using hierarchical clustering, and the colors in the heatmap represent the gene expression intensity. The hierarchical clustering analysis was conducted and heatmap was generated using GeneSpring 14.9 (Agilent Technology, Santa Clara, CA, USA), wherein clustering functionality and Euclidean correlation served as the distance measurement criteria. Additionally, Gene Ontology analyses were performed using the Fisher's exact probability test.

Statistical analysis

The clinicopathological characteristics of the patients are presented as median (range) for continuous variables and as number (%) for categorical variables. The differences in the proportion of CD44 + and CD24- cells between primary tumors and spheroids were analyzed using McNemar's Test. Statistical analyses were performed using JMP® version 14.0 (SAS Institute, Cary, NC, USA), and results with a p-value of < 0.05 were considered statistically significant.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions
All authors contributed to the study conception and design. SS, AK, and MI prepared the materials, and collected and analyzed the data. SS wrote the first draft of the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Declarations

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to T.K.

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