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Correlation of GD2 Biosynthesis Enzymes With Cancer Stem Cell Markers in Human Breast Cancer

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Abstract

Background/Aim: The disialoganglioside GD2 has been shown to promote cell proliferation, migration, tumor and metastasis through specific signaling pathways in tumor cells originating from the neuroectoderm, including melanomas, neuroblastomas, glioblastomas, and breast carcinomas. GD2 has therefore emerged as a potential diagnostic biomarker in early malignancy as evidenced by the high specificity of its expression in tumor cells. Furthermore, recent findings show that GD2 might also act as a novel cancer stem cell (CSC) marker. Our study aimed to investigate the relationship between GD2 and 34 recognized CSC markers in human breast cancer.

Materials and Methods: We analyzed the relationship between the mRNA expression profiles of three key enzymes involved in the biosynthesis of GD2 – B4GalT5, B4GALNT1, and ST8SIA1 – and 34 CSC markers in 91 human breast cancer tissue samples.

Results: All three enzymes had positive and statistically significant correlation between each other with $p<0.0001$. Furthermore, each enzyme was found to have highly significant correlations with 15 CSC markers associated with aggressive cancer behavior: BMI1, CX43, ALCAM (CD166), Podoplanin, CD29, CD24, CD49f, IL8RA, NGFR, hTERT, Nestin, OCT4, CTBP, PSCA and Myc.

Conclusion: These findings lend further support to the growing evidence that GD2 is a potential biomarker of CSCs and epithelial-mesenchymal transition (EMT) in human breast cancer that can be amenable to therapeutic targeting.

Keywords: GD2, GD3, gangliosides, breast cancer, cancer stem cells, epithelial-mesenchymal transition, EMT.

Introduction

Disialogangliosides (GDs) are glycosphingolipids that contain two sialic acid residues linked to the lactosylcera-mide (LacCer). They are found in the outer leaflet of the plasma membrane of developing embryonic cells and in the nervous system of healthy adults. The biosynthesis of gangliosides is mediated by specific enzymes namely glycosyltransferases

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and glycohydro-lases, which regulate the steady-state levels of gangliosides on the cell plasma membrane (1). Overexpression of disialogangliosides like GD3 or GD2 has been shown to activate cell signaling pathways leading to increased cell proliferation, migration, tumor growth and metastasis, especially in cancers of neuroectodermal origin, such as melanoma, neuro-blastoma, glioblastoma, or breast cancer (1-4). The high cancer tissue specificity of GD2 and GD3 indicates a potential role as diagnostic biomarkers in early cancer (1).

Figure 1 (5) illustrates the key enzymes involved in GD2 biosynthesis. The B4GalT5 gene encodes an enzyme that mediates the synthesis of LacCer, which is the starting point in the biosynthesis of all gangliosides. B4GALNT1, also known as GD2 synthase, encodes the enzyme that mediates the synthesis of gangliosides GM2/GD2. ST8SIA1 gene encodes GD3 synthase, which is considered the rate-limiting enzyme in GD2 biosynthesis and appears to play an important role in maintaining cancer stem cells (CSCs) and epithelial-to-mesenchymal transition (EMT) leading to a more aggressive biological behavior of malignancy (4-6). The pro-cancer effects of GD2 have been shown to involve the activation of c-Met and mTOR signaling pathways (2, 7).

Cancer stem cells (CSCs) are a small population of tumor cells capable of self-renewal, continuous proliferation and differentiation into various cancer cell types. These cells were initially identified in 1997 in hematological malignancies and subsequently in solid tumors. They are believed to persist within tumors contributing to clonal heterogeneity, tumor progression, metastasis, therapy resistance, and post-operative recurrence through their ability to generate new tumor cells. Moreover, CSCs can survive most conventional treatments and persist as dormant circulating tumor cells (CTCs) and/or disseminated tumor cells in distant organs (8). Therefore, targeting CSCs could offer novel therapeutic approaches to enhance the clinical outcomes of cancer patients (8).

Currently, there is no consensus regarding a universal marker for identifying CSCs across different tumor types. Therefore, a tumor-specific CSC phenotype can be defined by the co-expression of multiple intracellular and plasma membrane-associated markers. More recently, GD2 has been shown to be a more specific marker of breast CSCs and EMT state than conventional markers like CD44, which is also expressed by normal tissues (2, 4). However, GD2 expression was reported to correlate with that of CD44 in breast cancer models (2).

Direct detection and quantification of GD2 in tumor samples is challenging due to its degradable glycolipid nature, therefore measuring the key enzymes responsible for GD2 biosynthesis could represent a more accurate reflection of its expression (2). This study aimed to investigate the correlation between the mRNA expression of the genes encoding the 3 key enzymes responsible for GD2 synthesis, B4GalT5, B4GALNT1, and ST8SIA1, and the mRNA expression of a panel of 34 genes recognized as CSC markers or vital for the maintenance and survival of CSCs, using a cohort of 91 human breast cancer specimens.

Materials and Methods

Patients and tissue samples. All the breast cancer tissue samples used in the study conformed to all the institutional and ethical requirements for sample collection and analysis. Collectively, ninety-one breast cancer tissue samples were used in this study. The tumor samples were harvested shortly after surgical resection in a way that did not affect the histopathological evaluation of the tumor margins. Following harvesting and collection, the samples were immediately snap-frozen and stored at −80˚C until mRNA extraction and analysis. This study was approved by the Institutional Review Board and informed consent was obtained from all participants in the study.

Tissue processing: RNA extraction and cDNA synthesis. This part of the study involved a very careful RNA extraction process as well as the cDNA synthesis after which the analyses were conducted on the samples from each frozen tissue. The quality of these samples was highly controlled by quantifying β-actin mRNA that was used as a calibrator for RNA level at 250 ng. Frozen tissue sections were cut at a thickness of 5-10 μm and kept for histopathology

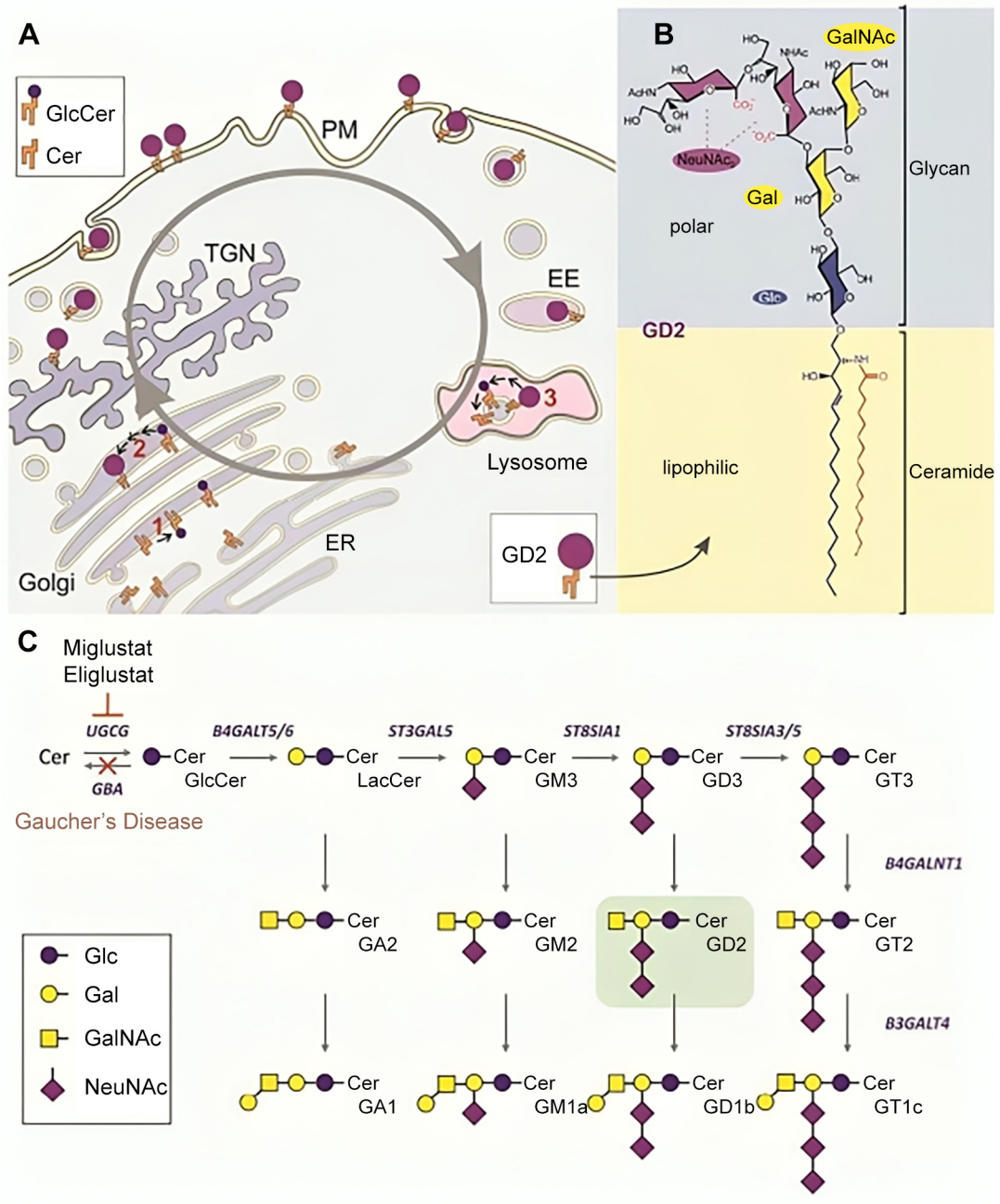

Figure 1. *Biosynthesis pathway of GD2 (5). A: Intracellular trafficking of GD2 and related glycosphingolipids. B: Molecular structure of GD2. C: Biosynthetic pathway of GD2 and related gangliosides.*

analysis. RNA extraction from 15-20 sections was performed using Trizol solution (Sigma-Aldrich Ltd, Poole, Dorset, UK). Then sections were gently mixed with an equal volume of ice-cold RNA extraction buffer using an electronic hand-held homogenizer. The amount of RNA obtained from the samples was very carefully measured by using UV spectrophotometry. After this, reverse transcription was conducted using the First Strand cDNA

Synthesis Kit for RT-PCR (AMV) (Sigma-Aldrich). This kit has an anchored oligo (dT) primer which has been selected to allow the generation of cDNA from 1 μg of total RNA in a 96-well plate. The quality of the obtained cDNA was assessed using β-actin primers as an internal positive.

Quantitative RT-PCR analysis. Real-time quantitative PCR (qPCR) based on the Amplifluor Technology was employed to accurately determine transcript levels in the cDNA library. The PCR primers were designed with Beacon Designer software (Palo Alto, CA, USA), which incorporated the Z sequence (5'-ACTGAACCTGACCGTACA-3') into one of the gene specific primers and were synthesized by Sigma-Aldrich. A custom made hot-start Master-mix designed for use in quantitative PCR was obtained from Abgene (Surrey, UK). This specific Master-mix was important for increasing the specificity and sensitivity of the PCR reactions and thus the quality of the results observed in the experiments. The FAM-tagged probe, Uniprimer, was supplied by Intergen Inc. (Oxford, UK). Detailed information about the primers used in our study, including their sequences and specific targets, is provided in Table I.

To maximize the relevance and reliability of the results obtained through the qPCR technique, quantitation of the transcripts was performed relative to a standard plasmid with known quantity. For each PCR run, there was a negative and a positive control to check validity of the results. Distilled water was used as a negative control to determine presence of contamination while cDNA obtained from a blend of cancerous breast tissue was used as a positive control to evaluate the efficiency and specificity of the PCR amplification. In addition, transcript levels obtained from the qPCR analysis were further normalized against cytokeratin 19 (CK19) expression. This normalization step was essential to correct for varying amounts of epithelial tissue between the different samples, ensuring that the results were not skewed by differences in sample composition.

Statistical analysis. Statistical analysis was performed using the statistical package computer program SPSS Version 29. To assess the correlation between the investigated variables, Spearman's rank correlation coefficients were computed. The level of statistical significance was assessed with two-sided *p*-value, using an alpha level of 0.05.

Results

The data revealed that all three genes involved in GD2 synthesis demonstrated a significant correlation with one another, with *p*-values less than 0.0001 (Figure 2). In addition, all three GD2 biosynthesis enzymes exhibited highly significant correlations with 15 out of the 34 CSC markers analyzed (Table II). These markers include BMI1 (Figure 3A), CD29 (Figure 3B), CD49f (Figure 3C), CD24 (Figure 4A), hTERT (Figure 4B), ALCAM (CD166) (Figure 4C), CX43 (Figure 5A), IL8RA (Figure 5B), Podoplanin (Figure 5C), NGFR (Figure 6A), Myc (Figure 6B), Nestin (Figure 6C), OCT4 (Figure 7A), PSCA (Figure 7B), and CTBP (Figure 7C). The correlation plots for these markers are illustrated in Figure 3, Figure 4, Figure 5, Figure 6, Figure 7, and Figure 8, which provide a visual representation of the relationships between GD2 biosynthesis and various CSC markers.

However, no significant correlations were observed between any of the three GD2 biosynthesis enzymes and 18 CSC markers including some traditional epithelial CSC markers, such as CD34 (Figure 8A), CD133 (Figure 8A), CD44, and ALDH. This indicates that the GD2 synthesis genes do not have a significant association with these commonly studied epithelial CSC markers, suggesting that their role in CSC biology may be distinct from that of the traditional markers. Finally, Notch2 exhibited significant negative correlations with B4GalT5 (p=0.024) and B4GALNT1 (p=0.046).

Discussion

Our study demonstrated highly significant positive correlations between GD2 synthesizing enzymes and key breast cancer stem cell (BCSC) markers: BMI1, CX43, ALCAM (CD166), Podoplanin, CD29, CD24, CD49f, IL8RA,

Table I. *Primer sequences used in the study.*

Gene name	Forward (5' – '3)	Reversed (5' – '3)
ALCAM	AGGAGGTTGAAGGACTAAA	ACTGAACCTGACCGTACAGGGATCAGTTTTCTTTGTCA
MET	ACTGAACCTGACCGTACAGAGCCAAAGTCCTTTCAT	ATCGAATGCAATGGATGAT
STAT5	TACAATGAACAGAGGCTGGT	ACTGAACCTGACCGTACAATGTCTGGTTGATCTGAAGG
IL8RA	TGGGGACTGTCTATGAATCT	ACTGAACCTGACCGTACACATTTCCCAGGACCTCATA
NGF	AGACTGTCACCATCTCCAAG	ACTGAACCTGACCGTACACATCTTCTCCTGTTTCTTGC
NGFR	GCCTACATAGCCTTCAAGAG	ACTGAACCTGACCGTACATGGAGTTTTTCTCCCTCTG
Podoplanin	GAATCATCGTTGTGGTTATG	ACTGAACCTGACCGTACACTTTCATTTGCCTATCACAT
CK19	CAGGTCCGAGGTTACTGAC	ACTGAACCTGACCGTACACAGTTTCTGCCAGTGTGTCTTC
CD24	AACTAATGCCAACCACCAAG	ACTGAACCTGACCGTACATAAGAGTAGAGATGCAGAAGAG
CD29	CCTGCCTTGGTGTCTGTG	ACTGAACCTGACCGTACACCTGTGTGCATGTGTCTTTC
CD34	TCAGCAAAGTGGAAGTTAT	ACTGAACCTGACCGTACAGTAGTTTGGGAATAGCTCTG
CD44	ACCATGGACAAGTTTTGGTGGCA;	ACTGAACCTGACCGTACACTGTAGCGACCATTTTTCTC
CD49	GCGAGCCTTCATTGATGTG	ACTGAACCTGACCGTACACTACAGTCTTTGAGGGAAACAC
CD49b	GCCTGCAGAAGAATATGGTA	ACTGAACCTGACCGTACATCCAGACTGATGTCCACAC
CD133	GCAAATGTGGAAAAACTGAT	ACTGAACCTGACCGTACATTAAATAGCTTCCCAGAGAGA
PSCA	ATGAAGGCTGTGCTGCTT	ACTGAACCTGACCGTACAAGTCCTCGTTGCTCACCT
TSA	CTTGAACCAGAAGAGCAATC	CTGAACCTGACCGTACACACTAGCAGACACAGTCACG
B4GALNT1	CCAGATCTTGCTCCTGAG	ACTGAACCTGACCGTACACCACTGGACTCACAACTG
B4GALT5	TGCTGTACTTCGTCTATGTG	ACTGAACCTGACCGTACAATAAACCTGAGCACCGATTG
ST8Sia1	TGGTATGACGGGGAGTTTT	ACTGAACCTGACCGTACACTTGACAAAGGAGGGAGATT
SLUG1	CTCCAAAAAGCCAAACTACA	ACTGAACCTGACCGTACAGAGGATCTCTGGTTGTGGTA
SNAIL	TCTTTCCTCGTCAGGAAGC	ACTGAACCTGACCGTACACTGCTGGAAGGTAAACTCTG
TWIST	AAGCTGAGCAAGATTCAGAC	ACTGAACCTGACCGTACAGAGGACCTGGTAGAGGAAGT
Notch1	GGGCTAACAAAGATATGCAG	ACTGAACCTGACCGTACAGTTGGCAAAGTGGTCCAG
Notch2	AAGAAACAGAGGATGACACG	ACTGAACCTGACCGTACATGGTCTGAGTCTTGAACACA
BMI1	TGTGTGCTTTGTGGAGGGTAC	ACTGAACCTGACCGTACATGGTCTGGTCTTGTGAACTTGG
Nestin	CCCGTACCCCTACCTTGG	ACTGAACCTGACCGTACAGGCTCTGATCTCTGCATCTAC
OCT4	CGCCGTATGAGTTCTGTG	ACTGAACCTGACCGTACAGGCTGAGAGGTCTCCAAG
CtBP	TACAGCGAGCAGGCATCC	ACTGAACCTGACCGTACATGGTCCTTGTTGACACAGTTC
Lamin-A	AAGCTTCGAGACCTGGAG	ACTGAACCTGACCGTACAATCTCCCGCTCCTTTTC
Lamin-B receptor	TGGGTGATCTCATCATGG	ACTGAACCTGACCGTACACTTCTCGGTGGACAAGC
Neutroplin-1	TCAACTTCAACCCTCACTTC	ACTGAACCTGACCGTACAAACTTTCCCCTAAAATGTCC
Neutroplin-2	TCAACCCTCACTTTGAAATC	ACTGAACCTGACCGTACAGATGTTCCCACAGTGTTTG
L1CAM	CCACTTGTTTAAGGAGAGGA	ACTGAACCTGACCGTACAGATGATGGCACTCACAAAG
ALDHA2	CACCACTCAGTGCACTCTAC	ACTGAACCTGACCGTACAACACCACTCTGGATGAGTTC
CX43	GGTTCAAGCCTACTCAACTG	ACTGAACCTGACCGTACAGTTACAACGAAAGGCAGACT
ALDHA1	TATCCTTGGAAATCCTCTGA	ACTGAACCTGACCGTACATTCTTTCTTCCCACTCTCAA
hTERT2	GTGGATGATTTCTTGTTGGT	ACTGAACCTGACCGTACAAGGTGAGACTGGCTCTGAT
LGR5	TTGACTTTGAGGAAGACCTG	ACTGAACCTGACCGTACAGTCCACACTCCAATTCTGAT
cMYC	TGCTCCATGAGGAGACAC	ACTGAACCTGACCGTACATGATCCAGACTCTGACCTTT

ALCAM: Activated leukocyte cell adhesion molecule; ALDHA2: aldehyde dehydrogenase 2 family member; B4GALNT1: beta-1,4-n-acetyl-galactosaminyltransferase 1; B4GALT4: beta-1,4-galactosyltransferase 4; BMI1: BMI1 polycomb ring finger proto-oncogene; CD: cluster of differentiation; CK19: cytokeratin-19; cMYC: Myc proto-oncogene protein; CtBP: C-terminal binding protein 1; CX43: connexin 43; hTERT2: human telomerase reverse transcriptase 2; IL: interleukin; L1CAM: L1 cell adhesion molecule; LGR5: leucine rich repeat containing G protein-coupled receptor 5; MET: MET proto-oncogene; NGFR: nerve growth factor receptor; NGF: nerve growth factor; Notch1: Notch receptor 1; Notch2: Notch receptor 2; OCT4: octamer-binding protein 4; PSCA: prostate stem cell antigen; SLUG: Slug homolog, zinc finger protein; SNAIL: Snail family transcriptional repressor 1; ST8Sia1: ST8 alpha-n-acetyl-neuraminide alpha-2,8-sialyltransferase 1; STAT5: signal transducer and activator of transcription 5A; TSA: thymic shared antigen-1; TWIST: twist basic helix-loop-helix transcription factor.

NGFR, hTERT, Nestin, OCT4, CTBP, PSCA, and Myc. These markers play critical roles in the maintenance, survival, and aggressive behavior of BCSCs.

BMI1, a member of the polycomb group (PcG), is a master regulator of cancer cell stemness, promoting cell proliferation, apoptosis, metastasis, and chemoresistance.

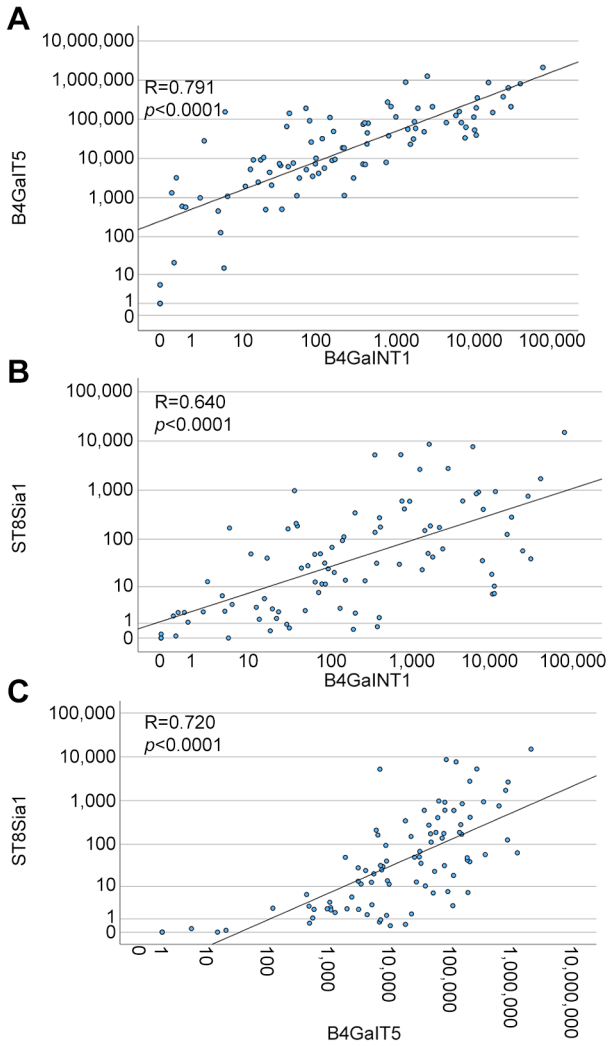

Figure 2. *Scatter plots and Spearman's rank correlation coefficients (r) between the expression levels of the GD2 biosynthesis synthesis enzymes. (A) B4GalT5 vs. B4GalNT1, (B) ST8Sia1 vs. B4GalNT1, and (C) ST8Sia1 vs. B4GalT5.*

Table II. *Statistically significant correlations (Spearman coefficients) between the GD2 synthesizing enzymes and 15 BCSCs.*

BCSC marker	B4GalNT1 r (*p*-Value)	B4GalT5 r (*p*-Value)	ST8Sia1 r (*p*-Value)
CD29	0.598 (<0.001)	0.706 (<0.001)	0.498 (<0.001)
CD49F	0.614 (<0.001)	0.808 (<0.001)	0.699 (<0.001)
CD24	0.380 (<0.001)	0.374 (<0.001)	0.457 (<0.001)
BMI1	0.721 (<0.001)	0.777 (<0.001)	0.757 (<0.001)
Nestin	0.675 (<0.001)	0.713 (<0.001)	0.642 (<0.001)
OCT4.	0.793 (<0.001)	0.871 (<0.001)	0.700 (<0.001)
PSCA	0.226 (0.032)	0.298 (0.004)	0.280 (0.008)
hTERT	0.545 (<0.001)	0.751 (<0.001)	0.613 (<0.001)
CTBP	0.333 (0.001)	0.282 (0.007)	0.424 (<0.001)
ALCAM	0.477 (<0.001)	0.612 (<0.001)	0.257 (0.017)
CX43	0.442 (<0.001)	0.581 (<0.001)	0.333 (0.001)
IL8RA	0.473 (<0.001)	0.494 (<0.001)	0.221 (0.041)
MYC	0.379 (<0.001)	0.454 (<0.001)	0.313 (0.003)
Podoplanin	0.618 (<0.001)	0.602 (<0.001)	0.586 (<0.001)
NGFR	0.434 (<0.001)	0.457 (<0.001)	0.419 (<0.001)

CD: Cluster of differentiation; BMI1: BMI1 polycomb ring finger proto-oncogene; OCT4: octamer-binding protein 4; PSCA: prostate stem cell antigen; hTERT2: human telomerase reverse transcriptase 2; CTBP: C-terminal binding protein 1; ALCAM: activated leukocyte cell adhesion molecule; CX43: connexin 43; IL: interleukin; NGFR: nerve growth factor receptor.

Its non-coding RNA further contributes to cancer progression by regulating the cell cycle, proliferation, and differentiation (9). CX43, encoded by the GJA1 gene, regulates tumor cell communication through tunneling nanotubes, facilitating CSC activity that impacts growth, differentiation, chemotherapy resistance, and environmental interactions (10, 11).

Octamer-binding transcription factor 4 (OCT4) is a pluripotency factor critical for CSC maintenance and differentiation. Its isoforms – OCT-4A, OCT-4B, and OCT-4B1 – exhibit distinct roles in normal stem cell functions and cancer progression (8, 12). Similarly, hTERT, the regulatory subunit of telomerase, is essential for telomerase reactivation during malignant transformation, predicting overall and disease-free survival while promoting EMT, stemness, metastasis, and recurrence (13-15).

Podoplanin enhances tumor progression, EMT, migration, invasion, metastasis, and inflammation, thus maintaining cancer stemness (16). IL8RA (CXCR1), a receptor for interleukin-8 (IL-8), activates pathways like Stat3/NF-κB and MAPK, driving CSC self-renewal *via* IL-8-dependent feedback loops (17). CD29 (integrin β1) and CD49f (α6-integrin) are crucial for adhesion to the extracellular matrix, CSC function, and disease progression, with CD49f also associated with EMT and poor prognosis (18, 19).

CD24, often studied in conjunction with CD44, CD133, or EpCAM, contributes to tumor aggressiveness,

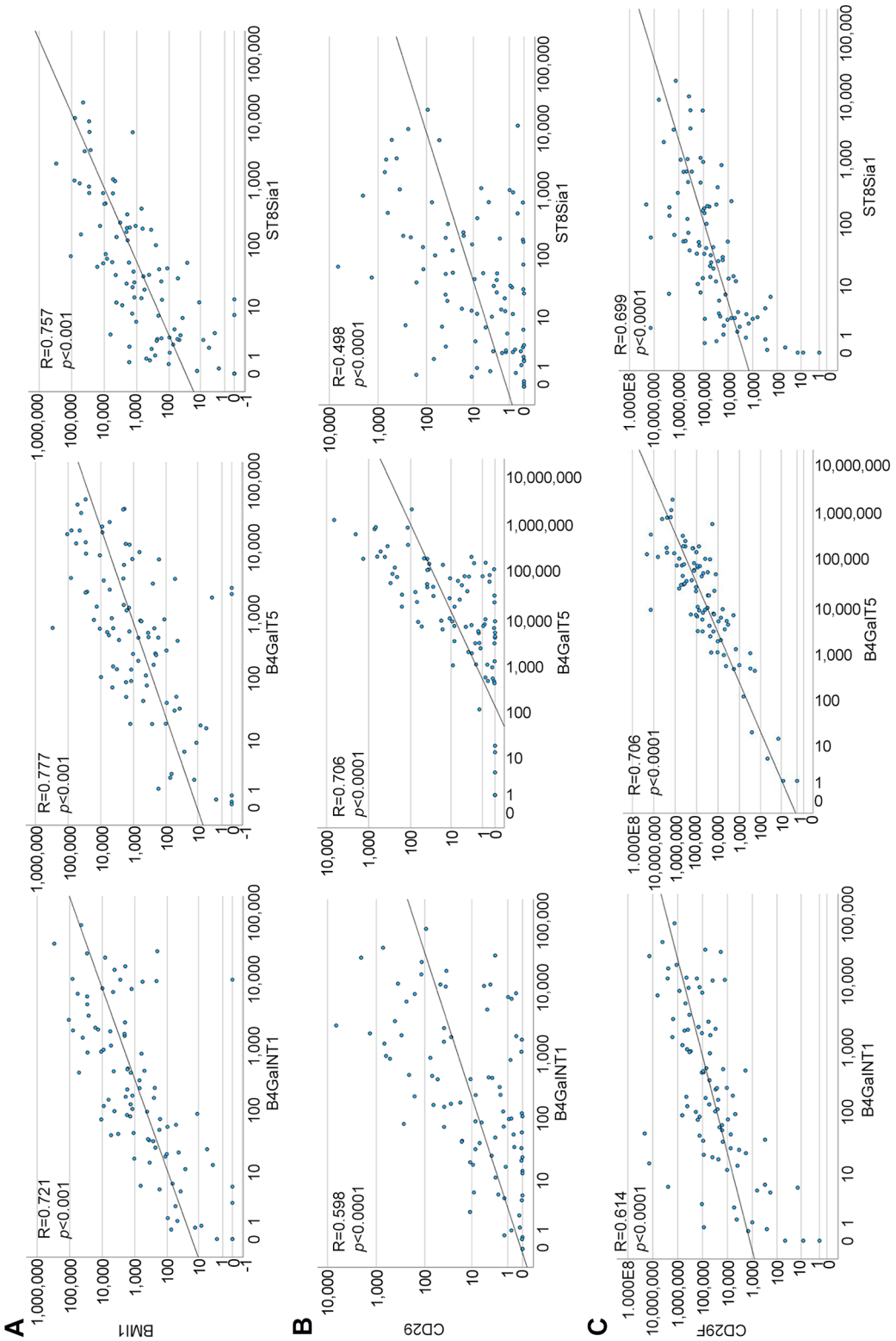

Figure 3. *Spearman's rank correlation between the mRNA expression of GD2 biosynthesis enzymes (B4GalNT1, B4GalT5, ST8Sia1) and BMI (A), CD29 (B) and CD49F(C).*

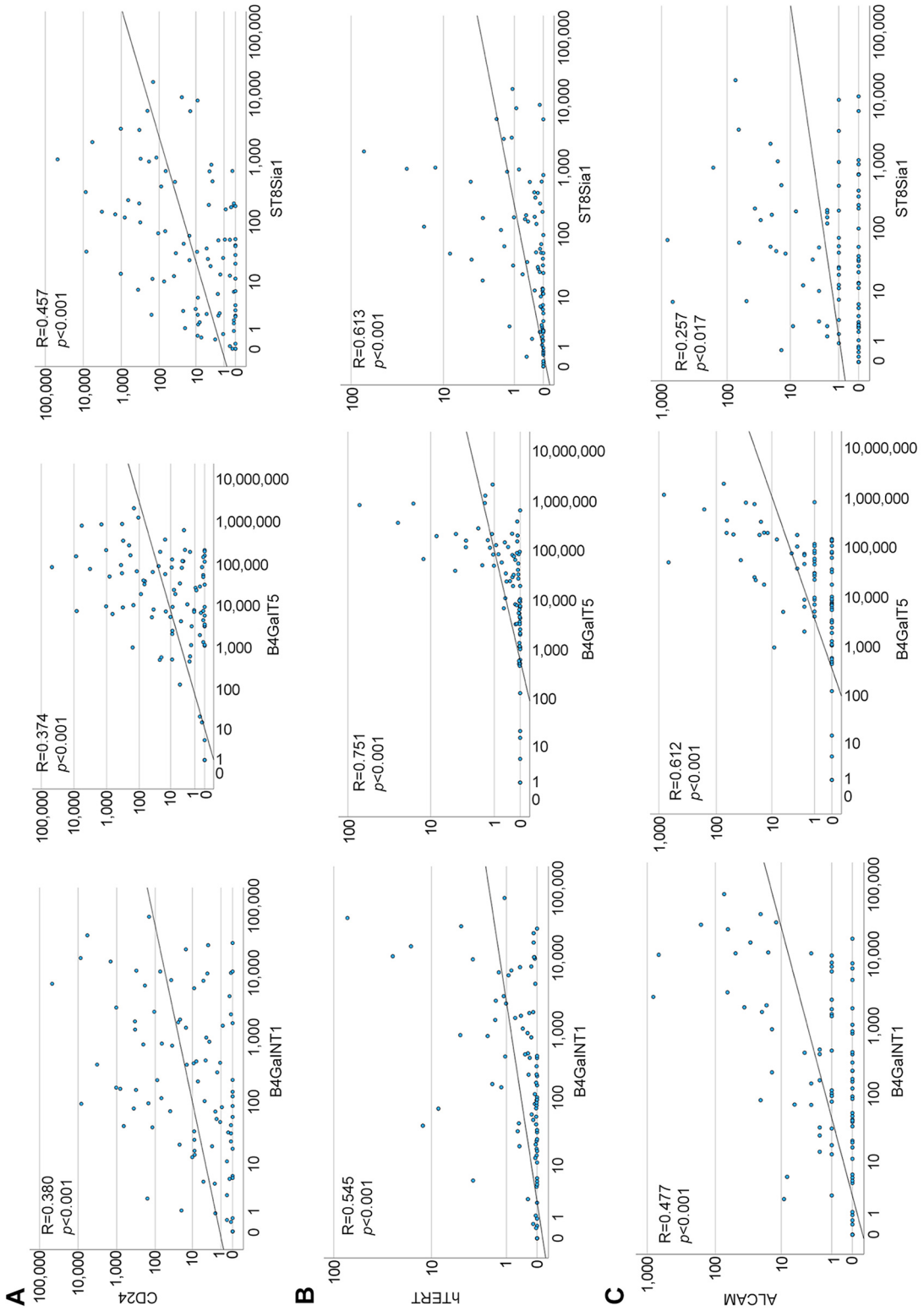

Figure 4. *Spearman's rank correlation between the mRNA expression of GD2 biosynthesis enzymes (B4GalNT1, B4GalT5, ST8Sia1) and CD24 (A), hTERT (B) and ALCAM (C).*

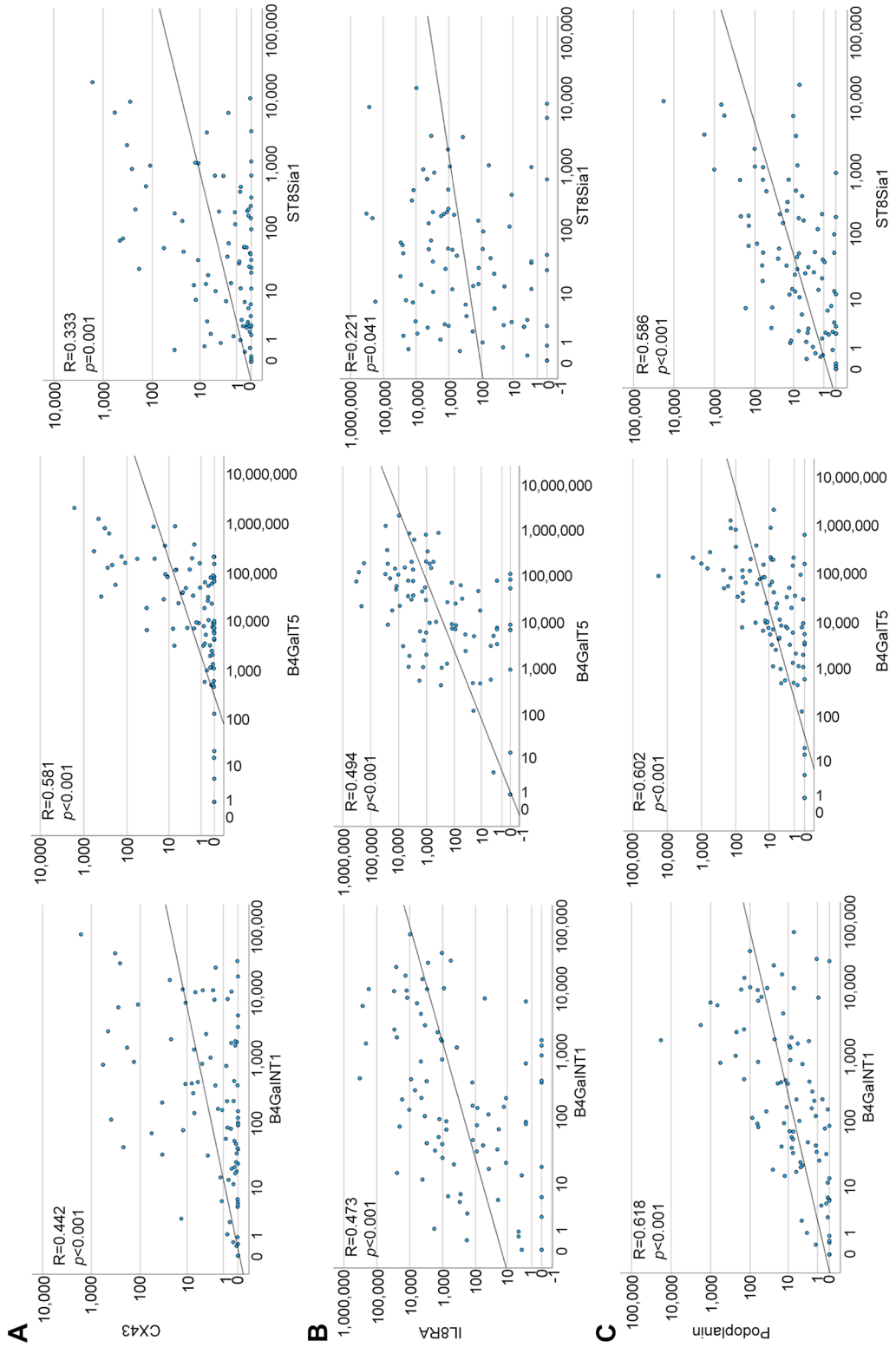

Figure 5. *Spearman's rank correlation between the mRNA expression of GD2 biosynthesis enzymes (B4GalNT1, B4GalT5, ST8Sia1) and CD24 (A), hTERT (B) and ALCAM (C).*

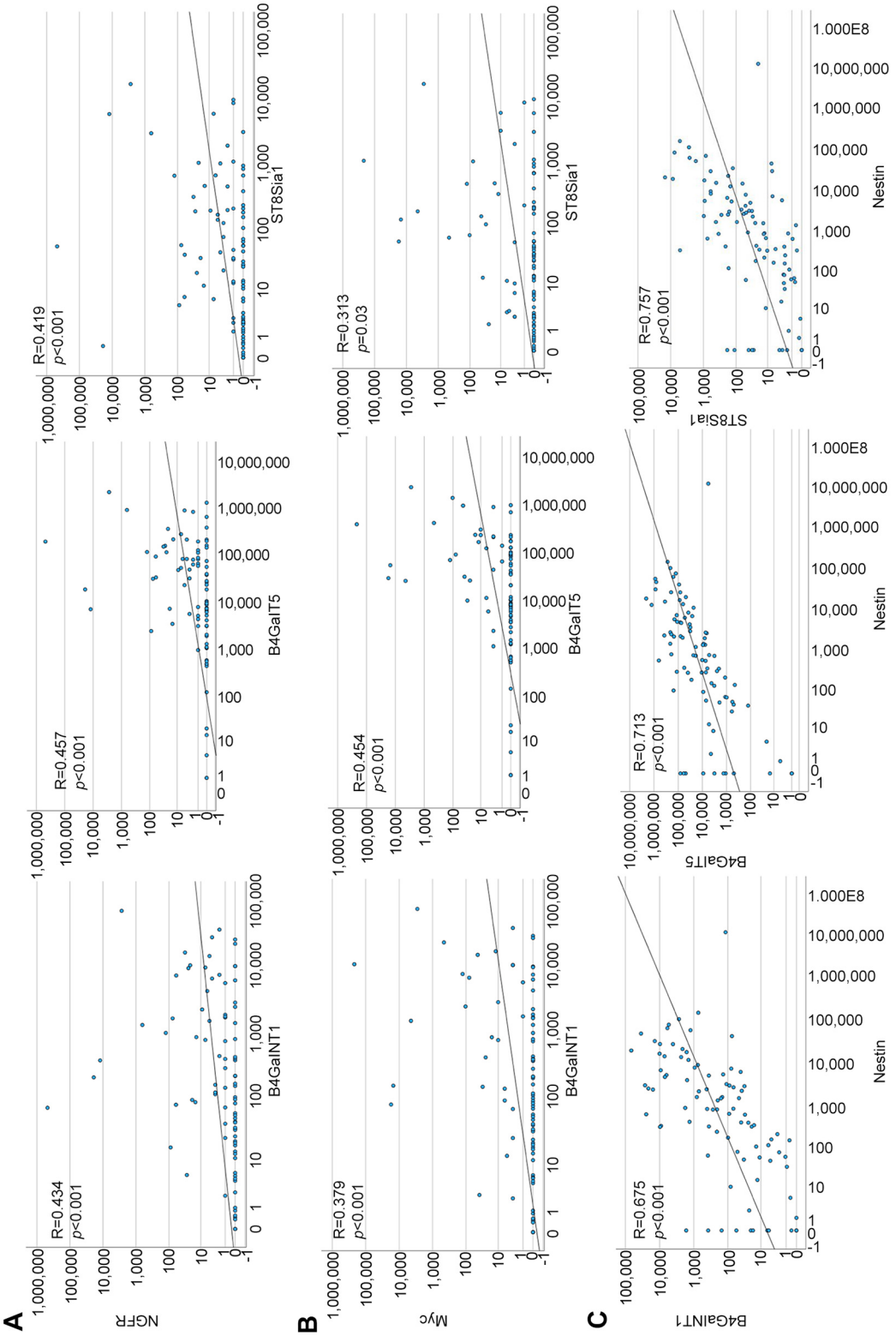

Figure 6. *Spearman's rank correlation between the expression of GD2 biosynthesis enzymes (B4GalNT1, B4GalT5, ST8Sia1) and NGFR (A), Myc (B) and Nestin (C).*

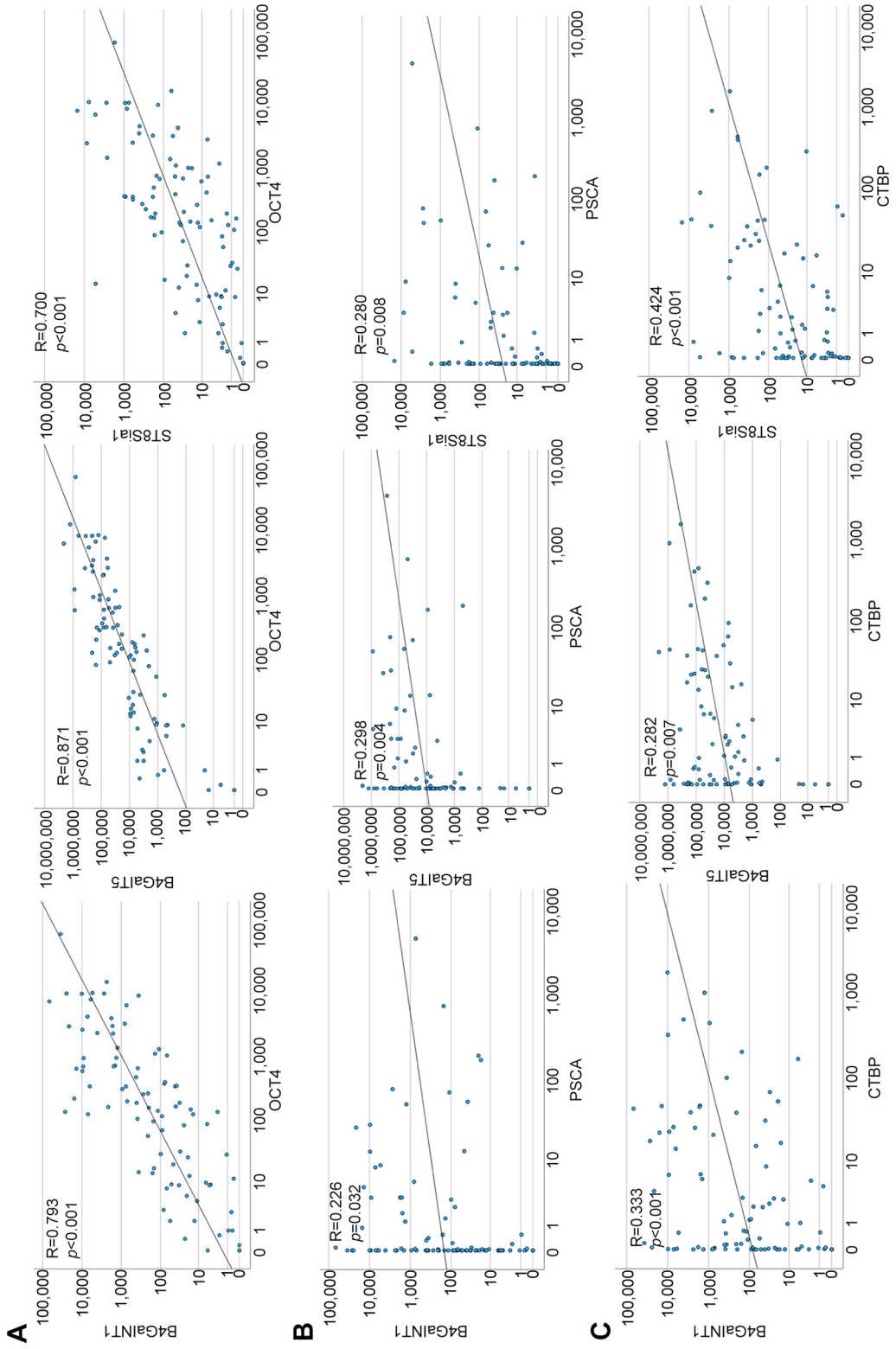

Figure 7. *Spearman's rank correlation between the expression of GD2 biosynthesis enzymes (B4GalNT1, B4GalT5, ST8Sia1) and OCT4 (A), PSCA (B) and CTBP (C).*

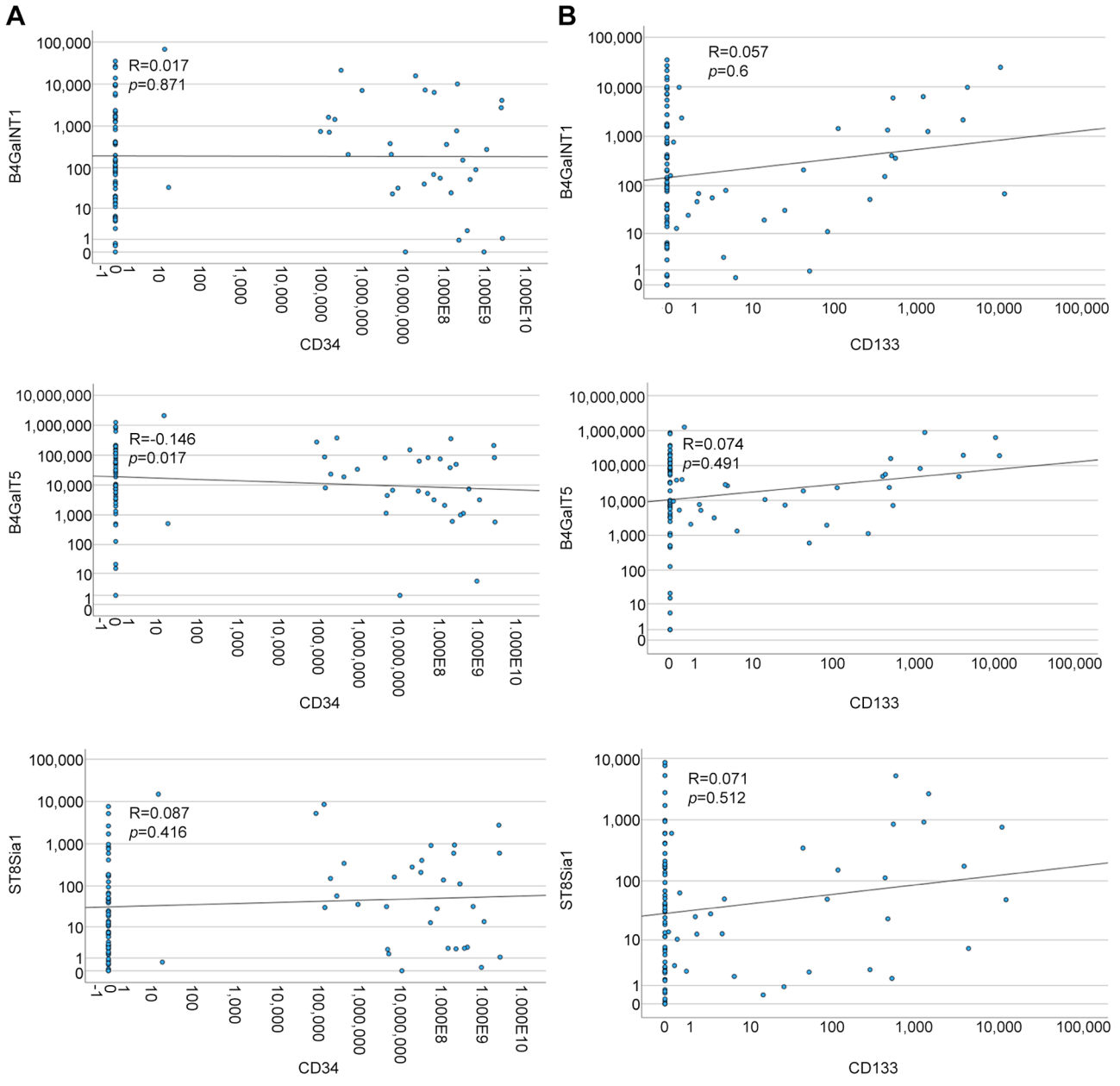

Figure 8. *Spearman's rank correlation between the expression levels of GD2 enzymes and CD34 and CD133 markers. (A) No significant correlations were observed between CD34 and B4GalNT1 (top), B4GalT5 (middle), or ST8Sia1 (bottom). (B) No significant correlations were observed between CD133 and B4GalNT1 (top), B4GalT5 (middle), or ST8Sia1 (bottom).*

metastasis, and therapy resistance. CD24-positive cells exhibit CSC phenotypes *via* JAK2–STAT3 signaling pathways (20). ALCAM (CD166) facilitates tumor progression, with its alternative splicing influencing cell adhesion and metastasis. Circulating ALCAM levels correlate with tumor progression (21).

NGFR (CD271) is a marker of CSC subpopulations, driving tumor initiation, progression, chemoresistance, and

metastasis (22). MYC, a proto-oncogene and transcription factor, supports stemness, self-renewal, and intra-tumoral heterogeneity, contributing to cancer progression and treatment resistance (23). Nestin, an intermediate filament protein, is associated with tumor aggressiveness, chemoresistance, and poor prognosis in multiple cancers, including glioblastoma and breast cancer (24).

CTBP, a transcriptional regulator, promotes EMT and CSC phenotypes in cancers like breast and colorectal cancer. Its inhibition reduces CSC growth and self-renewal (25). PSCA, initially identified as a prostate cancer biomarker, is overexpressed in multiple malignancies, including breast cancer, where it correlates with Her2 expression and aggressive behavior (26).

In our study, GD2 enzymes showed no correlation with traditional BCSC markers such as CD44, CD133, and ALDH, which lack specificity for BCSCs due to their expression in normal cells (2). Battula *et al.* previously reported a correlation between GD2 and CD44 in experimental and human models (2). However, our findings diverged, as we observed no association between GD2 expression and key epithelial-to-mesenchymal transition (EMT) transcription factors such as Slug, Snail, and Twist, which are known to regulate CD44 expression and tumor suppressor pathways. Notably, we previously reported that reduced Snail expression was associated with poor outcomes and node-positive tumors (27), but this specific association was not observed with GD2 expression in the current study.

Interestingly, we observed a negative correlation between B4GalT5 and B4GALNT1 with Notch2. The CSC marker Notch plays complex roles in tumor biology, with the specific outcomes likely depending on the precise balance of its activity. Although Notch1 and Notch3 are considered pro-oncogenic, recent evidence suggests that Notch2 mediates breast cancer dormancy, and its high expression is associated with improved overall survival in both estrogen receptor-positive and estrogen receptor-negative breast cancer patients (28). Moreover, Capulli *et al.* demonstrated that breast cancer cells expressing high levels of Notch2 did not exhibit the typical characteristics of mammary CSCs but instead resembled hematopoietic stem cells (28).

Several studies reported that the GD2 expression, as measured by immunohistochemistry or immunofluorescence, was detected in most cases of human breast cancer, with rare expression in histologically normal breast tissues, supporting the notion that GD2 is a tumor-associated antigen (2, 29-33). Furthermore, most of these studies revealed a positive association between GD2 expression and triple negative breast cancer (TNBC) molecular subtype. Nevertheless, the studies reported conflicting results regarding the association between GD2 and prognostic parameters and clinical outcomes.

Therapeutic targeting of the GD2 system could potentially eliminate BCSCs which mediate cancer cell resistance to systemic and radiation treatment and disease recurrence (34). Monoclonal antibodies, chimeric antigen receptor-T cell therapy, and GD3 synthase inhibition offer multiple avenues for treatment, with ongoing research aiming to optimize these strategies for clinical use. By effectively targeting GD2, these therapies could significantly improve outcomes for breast cancer patients, particularly for those with treatment-resistant forms of the disease (35).

Naxitamab, a humanized moAB targeting GD-2 (Danyelza®, Y-mAbs Therapeutics, Inc.) has been recently approved for the treatment of high-risk refractory or relapsed neuroblastoma in bone marrow and/or bone in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) after successful phase 3 clinical trials (36). Dinutuximab was also approved for treating high-risk neuroblastoma in patients with minimal residual disease and more recently for those with refractory or resistant neuroblastoma. However, its use is limited by neuropathic pain, which can be dose-limiting (35-37). Furthermore, dinutuximab is less appealing than naxitamab in clinical practice due to the long period required for intravenous administration that hinders its use in the outpatient setting. Furthermore, the O-acetyl derivative of GD2, OAcGD2, is emerging as a novel CSC marker that can be therapeutically targeted with

monoclonal antibodies (37). The anti-OAcGD2 monoclonal antibody, mAb8B6, effectively eradicated OAcGD2+ cells and reduced tumor growth in a patient-derived xenograft (PDX) model, suggesting mAb8B6 as a promising immunotherapeutic agent for targeting BCSCs (37).

The strength of our study lies in the use of robust RT-PCR methodology to analyze the mRNA expressions of all 3 key enzymes responsible for GD2 biosynthesis and a large panel of CSC markers or associated genes in a significant number of human breast cancer samples. However, our investigation has limitations. We only measured mRNA transcript levels of the enzymes, without quantifying protein expression of GD2 biosynthesis enzymes. Additionally, we did not directly verify the presence of GD2 in BCSCs, making our evidence regarding the role of GD2 in BCSCs indirect, based on correlations with important BCSC markers and associated factors. GD2 is a ganglioside, which shares similarities with lipids, rather than being a protein or mRNA. Due to its nature, measuring GD2 directly is challenging and requires specialized techniques, such as thin-layer chromatography or immunofluorescence, which are not commonly performed in many laboratories. As a result, we measured the mRNA levels of the three key enzymes responsible for GD2 biosynthesis instead. Like other glycolipids, GD2 is stable only under specific conditions, such as low temperatures and proper solvents, but is sensitive to pH, temperature, and enzymatic degradation. In biological systems, enzymes can degrade GD2, requiring meticulous handling to maintain its stability during experiments. Therefore, our study focused on measuring the mRNA of the enzymes responsible for GD2 synthesis.

While our study might not provide direct functional evidence that GD2 is a definitive BCSC marker, the correlations we have found between GD2 and other recognized BCSC markers are valuable and lend further support to the recently emerging evidence that GD2 is a novel BCSC marker as initially suggested by Battula *et al.* in 2012 (2, 38). Further functional studies could build on this foundation to clarify the role of GD2 in BCSC biology.

Conclusion

Our results provide indirect evidence supporting the recently emerging concept that GD2 is a novel marker of BCSCs. By demonstrating that the expressions of GD2-synthesizing enzymes correlate with multiple established markers of BCSCs, our findings align with the notion that GD2 may play a role in identifying this specific cell population and is potentially involved in the stem-like properties of breast cancer cells, such as self-renewal and resistance to treatment.

Use of AI Statement

AI was used to check the English language grammar during the editing of the manuscript.

Conflicts of Interest

Kefah Mokbel has received honoraria for offering academic and clinical advice to Merit Medical and QMedical corporations.

Authors' Contributions

Conceptualization, N.O. and K.M; conceptualization and writing – original draft preparation, N.O, K.M, and W.J.; writing – review and editing, N.O., K.M, and M.R. visualization, N.O., K.M., M.R, W.J., A.L., and T.M.; funding acquisition, N.O. and K.M. All Authors have read and agreed to the published version of the manuscript.

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