RESEARCH ARTICLE

Editorial Process: Submission:10/07/2024 Acceptance:03/15/2025

Increased expression of *PTCH1* and *GLI1* in Bangladeshi TNBC Patients after Treatment with Doxorubicin and Cyclophosphamide Suggests a Favorable Response to Chemotherapy

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Abstract

Background: The Hedgehog (Hh) signaling pathway is commonly exploited for intercellular communication throughout development and organogenesis. Disruption of Hh signaling contributes to benign and malignant growth including breast cancer (BC). The distinct expression pattern of genes can be used as a sign of treatment efficacy and to make a new treatment plan. The present study was designed to evaluate the expression pattern of Hh signaling pathway genes such as Sonic Hedgehog (SHH), Smoothend (SMO), Glioma-associated Oncogene Homolog 1 (*GLI1*), and Patched 1 (*PTCH1*) in TNBC before and after chemotherapeutic treatment. **Methods:** A total of 18 (eighteen) TNBC patients were included in this study. Expression of the Hh signaling pathway genes was measured in the TNBC patient's tumor tissue and blood sample before and after chemotherapy by qRT-PCR. GraphPad Prism was used to analyze the experimental data. **Results:** SHH and SMO expression were found to increase in tissue samples of TNBC subtypes, whereas *GLI1* and *PTCH1* expression were decreased compared to adjacent noncancerous tissue. After chemotherapeutic treatment, upregulation was found for *GLI1* and *PTCH1* expression in blood samples when compared to the expression in blood samples of untreated-stage patients or healthy control group. Moreover, SHH and SMO expression were found to decrease compared to the untreated group. **Conclusion:** Upregulation of *GLI1* and *PTCH1* expression, with substantially decreased expression of SHH and SMO after the chemotherapeutic treatment may be associated with positive outcome of chemotherapy.

Keywords: Chemotherapy- Doxorubicin- Cyclophosphamide- Hh signaling- TNBC- Breast cancer- *PTCH1*- *GLI1*

Asian Pac J Cancer Prev, **26 (3)**, 949-958

Introduction

Breast cancer (BC) is the deadliest disease occurring due to the sequential accumulation of inherited and/or acquired somatic mutations which propel the transformation of normal breast or mammary cells to cancerous cells with uncontrolled and aggressive cell division properties. It is the most malignant type of cancer and is often diagnosed in women all over the world [1]. Ductal hyperproliferation is caused by both oncogenic and anti-oncogenic gene mutations, as well as aberrant amplification of that gene, and this genetic alteration can lead to malignant breast tumors or even metastatic carcinomas [2]. Human breast carcinomas are a dynamic illness with heterogeneous pathologies and molecular profiles that can initiate in different cell types (breast epithelial stem cells or their progeny and descendants). These differential levels of distinct molecular signatures contribute to differential sensitivity to treatment [3, 4]. Several types of tumors may develop within different areas of the breast with variable expression of oncogenes and/or tumor suppression genes which determine treatment strategy and tumor management. Several recent studies distinguished breast cancer based on pervasive differences in their gene expression patterns. According to gene expression pattern, breast cancer is subdivided into

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Luminal A, Luminal B, Human epidermal growth factor receptor 2 positive (Her2+), Basal-like and Normal breast-like [5, 6]. The Basal-like breast cancers (BLBC) have two distinguishing characteristics such as TNBC phenomenon (both ER, PR, and HER2 negative) and Basal-like (expresses high-molecular-weight/basal cytokeratin such as CK5/6, CK14, and CK17 and/or HER1) [7]. After activation of different cancer signaling pathways, breast cells grow recalcitrant into benign (not dangerous to health) or malignant (potential to be dangerous). Benign cells are supposed to be normal in appearance with a slow growth rate and have minimal potential for spread, and they stay at their primary location in the case of breasts. On the other hand, malignant cells are intractable with excessive growth rate and eventually, they metastasize beyond their primary location to distant parts of the body [8]. Different embryonic signaling pathways, such as the Notch, Wnt, Hedgehog (Hh), BMP, and transforming growth factor (TGF)-β pathways play pivotal roles in stem cell signaling during embryogenesis, and normal tissue development and maintenance. Deregulation of these embryonic signaling pathways leads to uncontrolled cell division in human cancers, including breast, pancreatic, and lung [9]. Disruption of Hh signaling contributes to benign and malignant growth [10]. The Hh protein binds to a 12-pass transmembrane protein, Patched (*PTCH)*, and inactivates the *PTCH1* receptor. Hh-*PTCH1* complex de-represses another protein SMO [11]. Active SMO then translocates to the primary cilium (PC) and enhances the release of the transcription factor GLI. The translocation of GLI family proteins (GLI-1,2 and 3) to the nucleus initiates a downstream signaling cascade [12]. Previous studies have reported that Hh signaling pathway is linked to various types of cancers [13, 14] through upregulation of SHH ligand. In the case of breast cancer, abnormal upregulation of SHH has been shown to drive tumor progression and induce changes in the tumor microenvironment [15]. However, several studies have reported conflicting findings regarding the expression of another Hh signaling molecule, *PTCH1*, in breast cancer, with evidence of both upregulation and downregulation [16, 17]. Several studies have highlighted the definite involvement of *GLI1* and *GLI2* in the pathogenesis of breast [15, 18, 19]. Higher expression of *GLI1* have been reported in TNBC and basal-like breast cancer cell lines, as well as in TNBC tumors, whereas lower *GLI1* expression has been observed in the T47D and MCF7 cell lines [20]. Overall, current research indicates that aberrant activation of the Hh signaling pathway regulates the cellular constituents within the breast tumor tumor microenvironment [21]. It is already evident that breast cancer patients respond differently against therapy based on molecular subtypes. TNBC subtypes are under more concern as they occur in typically younger patients' age with poorly differentiated tumor characteristics. Moreover, the survival rate of the patients is also lower because they do not benefit from targeted therapies [22-25, 26]. Because there is insufficient data on which to base treatment selection, no specific systemic treatment strategy is currently recommended for the treatment of TNBC. The most recommended antineoplastic drugs in Bangladesh include cisplatin, doxorubicin, cyclophosphamide, trastuzumab, 5-fluorouracil, docetaxel, and carboplatin. From patient screening data in Chittagong Medical College and Hospital (CMCH), Chattogram, Bangladesh, we observed that doxorubicin (DOX) and cyclophosphamide (CP) are commonly recommended in TNBC treatment. DOX and CP are generally recommended in combination with 5 fluorouracil (FAC regimen). DOX, a natural anthracycline antibiotic, is one of the most effective chemotherapy drugs used against a broad spectrum of solid tumor types [27]. CP, a nitrogen mustard with anti-neoplastic properties, works by alkylating DNA. Efficient chemotherapy must have effects on embryonic genes and chemotherapy may increase tumor suppressor gene's expression and reduce oncogenic expressions aiming to enhance survivability. Our laboratory is researching such dysregulated embryonic signaling pathways. Sonic Hedgehog (SHH) signaling pathway is one of them. We have found altered SHH expression patterns in TNBC patients of Bangladesh [28]. Since the survival rate is inferior, different treatment strategies are used to treat breast cancer patients. All the drugs mediate changes in different molecular signaling. The distinct expressional pattern of genes can be used as a sign of treatment efficacy and to make a new treatment plan. Moreover, confirming therapeutic response and efficacy is highly demanded to avoid overtreatment. Due to the cellular and molecular heterogeneity of breast cancer, patients may respond differently to therapy implying that subtype-specific information is important to confirm prognosis and prediction. Recent advancements in gene expression profiling hold the promise of providing new avenues for forecasting and predicting breast cancer outcomes. Keeping all in mind, we have checked the expression pattern of Hh signaling molecules such as SMO, *PTCH1*, and *GLI1* in TNBC patients when treated with DOX and CP and found a significant change in the expression pattern of these targeted molecules in different chemotherapeutic cycles.

Materials and Methods

Selection of study subjects

We have hit upon a plan to investigate gene expression patterns in chemotherapeutic breast cancer patients from January 2022 to December 2022. We have screened 39 patients with breast cancer. After doing immunohistochemistry and histopathology, we have discarded 21 patients due to different subtypes than TNBC, and we collected tumor tissue and surrounding normal tissue from 18 TNBC patients to conduct this research work. The age range of the selected patients was 35 to 55 years. We have selected patients based on several criteria: who have unilateral breast cancer, have not undergone mastectomy or breast-conserving surgery, have no other concomitant disease, received no chemotherapy before starting our research, and patients with grade II stage. We collected blood samples from 11 TNBC patients (we could not attend all the 18 patients due to some unavoidable circumstances) before starting their chemotherapy cycle and after every two cycles of chemotherapy up to six cycles. We also collected blood

from 10 healthy female volunteers of a similar age range to patients in our study as a control. Doxorubicin (Doxil, Kobaltweg 49, Netherlands)) and cyclophosphamide (Endoxen, Kobaltweg 49, Netherlands) were used to treat the disease burden.

Study area

All the patient data and samples were collected retrospectively from Chittagong Medical College and Hospital (CMCH), Chattogram, Bangladesh. Histopathology and immunohistochemistry tests have been done by expert pathologists. Subsequent molecular analyses were performed at the Centre for Research Excellence (CRE), Department of Biochemistry and Molecular Biology, University of Chittagong, Chattogram, Bangladesh.

Collection of tissue and blood samples from TNBC patients

In collaboration with the Surgery Department of Chittagong Medical College and Hospital (CMCH), Chattogram, tissue samples were collected during the surgery of the TNBC patients. RNAlater was used in the collection tube to preserve RNA stability and stored at -20°C freezer for a short time before isolating RNA. With the help of a certified phlebotomist, 5 ml of peripheral blood samples were withdrawn and immediately transferred to an EDTA anticoagulant tube and shipped to the research laboratory for further analysis. The blood samples were collected at different time intervals; i) before starting chemotherapeutic treatment (untreated sample), ii) 15 days after taking the 2nd chemotherapeutic cycle (cycle 2 sample), iii) 15 days after taking the 4th chemotherapeutic cycle (cycle 4 sample), and iv) 15 days after taking 6th chemotherapeutic cycle (cycle 6 sample). 5 ml blood was also collected from healthy female volunteers of a similar age range to patients as healthy control.

Histopathology of breast tumor samples

Normal tissue samples and malignant tumor samples were examined by histopathological techniques through microscopic identification. Histopathological techniques followed by grossing, tissue processing (tissues kept in alcohol, xylene), tissue impregnation (tissues kept in paraffin in a hot air oven at 40°C-50°C), embedding and blocking (using L-shaped block), section cutting by microtome machine, staining (by xylene, alcohol) and mounting by DPX solution. Typing, staging, and grading of breast cancer tissue samples were performed by histopathological examination by expert pathologists (our collaborator at CMCH).

Immunohistochemistry of breast cancer tissue samples

The immunohistochemistry technique was used to demonstrate the presence and location of breast cancer biomarkers ER, PR, and *HER2*. This technique was performed to demonstrate the presence and location of proteins in tissue sections. Immunohistochemical staining is accomplished with antibodies that recognize the target protein. As antibodies are highly specific, they will bind only to the protein of interest in the tissue section. The antibody-antigen interaction was then visualized using either chromogenic detection, in which an enzyme conjugated to the antibody cleaves a substrate to produce a colored precipitate at the location of the protein, or fluorescent detection, in which a fluorophore is conjugated to the antibody and can be visualized using fluorescence microscopy [29]. We used primary antibodies EP1, PgR636 & CB11 for the detection of ER, PR & HER2/neu respectively. The detection system was HRP (horseradish peroxidase) polymer-based. As per the scoring method for ER, PR expression in breast cancer, scores 0-2 are deemed negative while scores 3-8 indicated positive expression.

Isolation of RNA from tissue and blood sample

We followed the chloroform-isopropanol method to isolate mRNA. Before using TRIzol reagents, RBC lysis buffer was used to lyse red blood cells, leaving white blood cells behind. TRIzol reagent (supplied by Invitrogen Life Technologies; Cat No. 15596018) was added to the tissue sample and WBC pellet separately and vortexed. After that chloroform was added to the homogenate, inverted, mixed well, and centrifuged for phase separation. Chloroform solution precipitates cell debris (Carbohydrate, protein, and fat) and RNA remains in the upper aqueous phase. This upper aqueous phase was separated carefully and precipitated to get the total RNA. Isopropyl alcohol was added to the preparation and centrifuged and then RNA pellets were washed with 75% ethanol and centrifuged. The RNA pellet was dissolved in the appropriate amount of RNase-free water. Then the RNA was quantified with spectrophotometer ND 2000 (Thermo Scientific, USA) stored at -80°C and used further for agarose gel electrophoresis and cDNA synthesis.

Agarose gel electrophoresis

1% agarose gel electrophoresis (AGE) was run for total RNA to determine its purity and integrity. We prepared 1µg or 1000 ng/µl RNA sample. Then we added loading dye & nuclease-free water to the RNA sample. After that, we loaded it into the gel & electrophoresed it at 115 V. After migration of RNA samples as far as 2/3 of the total length of the gel, we visualized the gel on a UV gel documentation system.

cDNA synthesis

The GoScriptTM Reverse Transcription System from Promega Corporation was used to synthesize cDNA from RNA. This system includes a reverse transcriptase, and a specialized set of reagents designed for efficient synthesis of first-strand cDNA optimized for quantitative PCR amplification. For synthesizing cDNA, the components Oligo (dT), total RNA, dNTP, and distilled water were added to a nuclease-free PCR tube and then heated mixture to 70°C for 5 min and quickly chilled on ice. After the preparation of 20 µl final volume, the preparation was incubated at 42°C for 60 min. The reaction was inactivated by heating at 70°C for 5 min. The cDNA was then used as a template for amplification in PCR.

Oligo designing for target-specific real-time PCR

Target-specific oligo was designed using the NCBI database and Integrated DNA Technologies (IDT) website. The primer sequences are shown in Table 1.

Quantitative Real-time PCR (qRT-PCR)

qRT-PCR was applied to determine the GAPDH and expression of target genes. To do this, the SYBR green-based GoTaq qPCR master mix (Promega) was utilized. The BIO-RAD RT-PCR machine (CFX96-Real-time system) was used to perform thermal cycling and continuous monitoring of fluorescence detection. The reaction was carried out in triplicate for each sample with the following reaction conditions: 95°C for 2 min, 95°C for 30 sec, 30 seconds at annealing temperatures, and then 40 cycles at 72°C for 30 sec, followed by a hold at 72°C for 7 min. The comparative CT value method ($\Delta\Delta$CT method) was adopted to analyze the data.

TCGA public data set analysis

We have checked for the expression pattern of Hedgehog (Hh) signaling pathway molecules SHH, SMO, *GLI1*, and *PTCH1* in primary tumor tissue and different subtypes using the TCGA dataset in the UALCAN platform. Transcript per million (TPM) was used to determine the level of gene expression profile [30].

Statistical analysis

GraphPad Prism statistical software was used for performing all statistical analyses. Differences between the breast cancer patients and the control group were assessed using two-tailed unpaired Student's t-tests. One-way ANOVA was done to check the differences among groups. The values with a $p<0.05$ were considered statistically significant.

Materials

EDTA and non-EDTA tube used for blood sample collection from breast cancer patients was collected from Qiagen. TRIzol reagent for RNA isolation, Reverse Transcriptase enzyme kit for cDNA synthesis, Taq Polymerase enzyme kit for regular PCR, and SYBR Green for qRT PCR master mix were purchased from Invitrogen, CA, USA and Promega, Madison, USA. Chloroform and ethanol were obtained from Merck, Darmstadt, Germany. Isopropanol was collected from BDH Chemicals Ltd, Merck House, UK. DNase-RNase-free Water was supplied by GIBCO, CA, USA; and RNAse-free water by Qiagen Science, Germantown Rd, USA. Ethidium bromide and agarose were purchased from Bioshop, Burlington, Canada for electrophoresis. DNA Marker was brought from Bio Basic Inc., Ontario, Canada.

Results

Expression pattern of Hh signaling pathway molecules SHH, SMO, PTCH1, and GLI1 in TNBC tumor tissue compared to surrounding normal tissue

Hedgehog (Hh) pathway activation is important in the mammary gland to ensure proper development and avoid tumor formation. It has been evident that the Hh pathway stimulates breast tumor tissues, and the expression levels of the key genes (*SHH, SMO, GLI1, GLI2, PTCH1*) were higher than those in normal tissues. Moreover, the protein interaction network revealed that SHH and GLI2 had a high degree of intersection [31], [20]. Here, we have checked the SHH, SMO, *PTCH1*, and *GLI1* gene expression patterns in breast tumor tissues of the TNBC subtype. Significantly lower expression of *PTCH1* and *GLI1* ($P<0.02$ and $P<0.005$ respectively), and higher expression levels of SHH and SMO ($p<0.0003$ and 0.005 respectively) were observed in TNBC breast tumor tissue (Figure 1).

Expression pattern of Hh signaling molecules SHH, SMO, PTCH1, and GLI1 in different subtypes of breast cancer in the TCGA public dataset

In a previous study, it was shown that the expression of SMO and *GLI1* in TNBC was significantly increased in comparison to other subtypes [20]. The Cancer Genome Atlas (TCGA), is a groundbreaking cancer genomics program that characterized over 20,000 primary cancer molecularly and matched normal samples spanning 33 cancer types. It is a widely accepted online database on different cancers. We used TCGA dataset to investigate *SHH, SMO, PTCH1*, and *GLI1* mRNA expression in breast cancer patients. Expression of the selected genes were analyzed across different breast cancer subtypes

Table 1. Oligo Primer Sequence of the Genes and Their Tm

Gene symbol	Primer	Sequence (5´→3´)	Tm (ºC)
SHH	F	AGGACCCGGTTTGATCTTCT	57.7
	R	CCATGTGACACAGACAACC	57.4
GLI1	F	GTGCAAGTCAAGCCAGAACA	55.9
	R	ATAGGGGCCTGACTGGAGAT	56.4
SMO	F	GGGAGGCTACTTCCTCATCC	56.2
	R	GGCAGCTGAAGGTAATGAGC	56.8
PTCH1	F	ACAAACTCCTGGTGCAAACC	56
	R	CTTTGTCGTGGACCCATTCT	55
GAPDH	F	CAGCCTCAAGATCATCAGCA	54.8
	R	TGTGGTCATGAGTCCTTCCA	55.7

F, forward primer; R, reverse primer; Tm, melting temperature

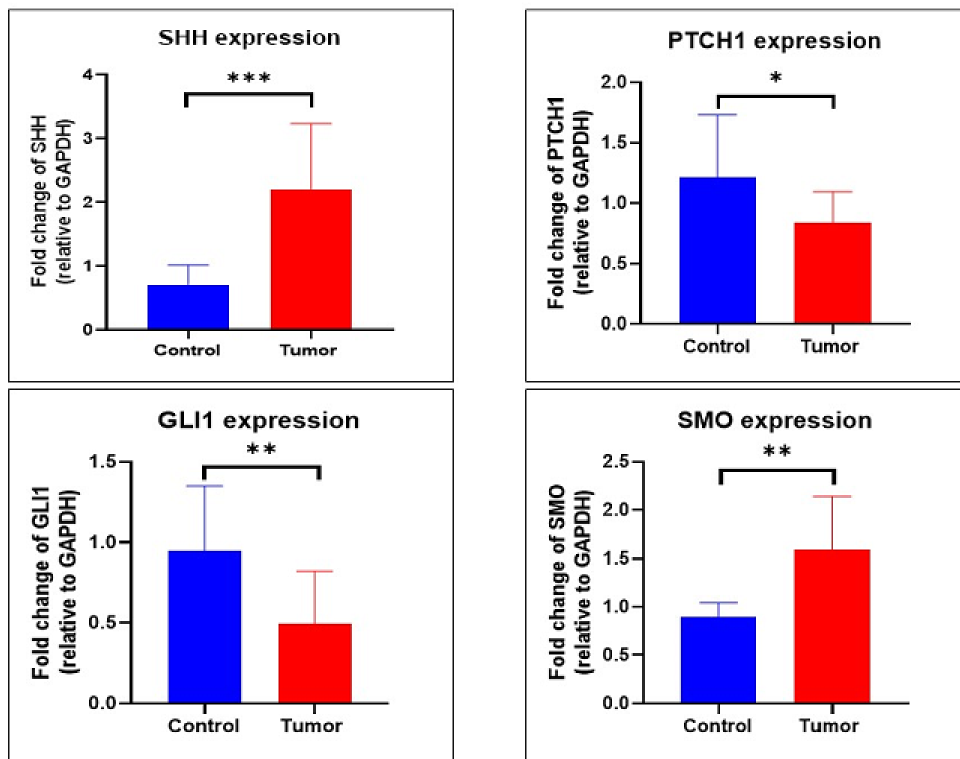

Figure 1. Expression Pattern of *SHH, SMO, GLI1*, and *PTCH1* in TNBC Tumor Tissue. qRT-PCR was utilized to determine gene expression patterns from cDNAs synthesized from tissue samples of the study cohort. Normalization with *GAPDH* expression was done to calculate relative expression. The data is presented here as mean ±SD. The number of TNBC tumor tissue samples was 18. *P<0.02, **P<0.005, ***P<0.0003. P value < 0.05 was considered statistically significant.

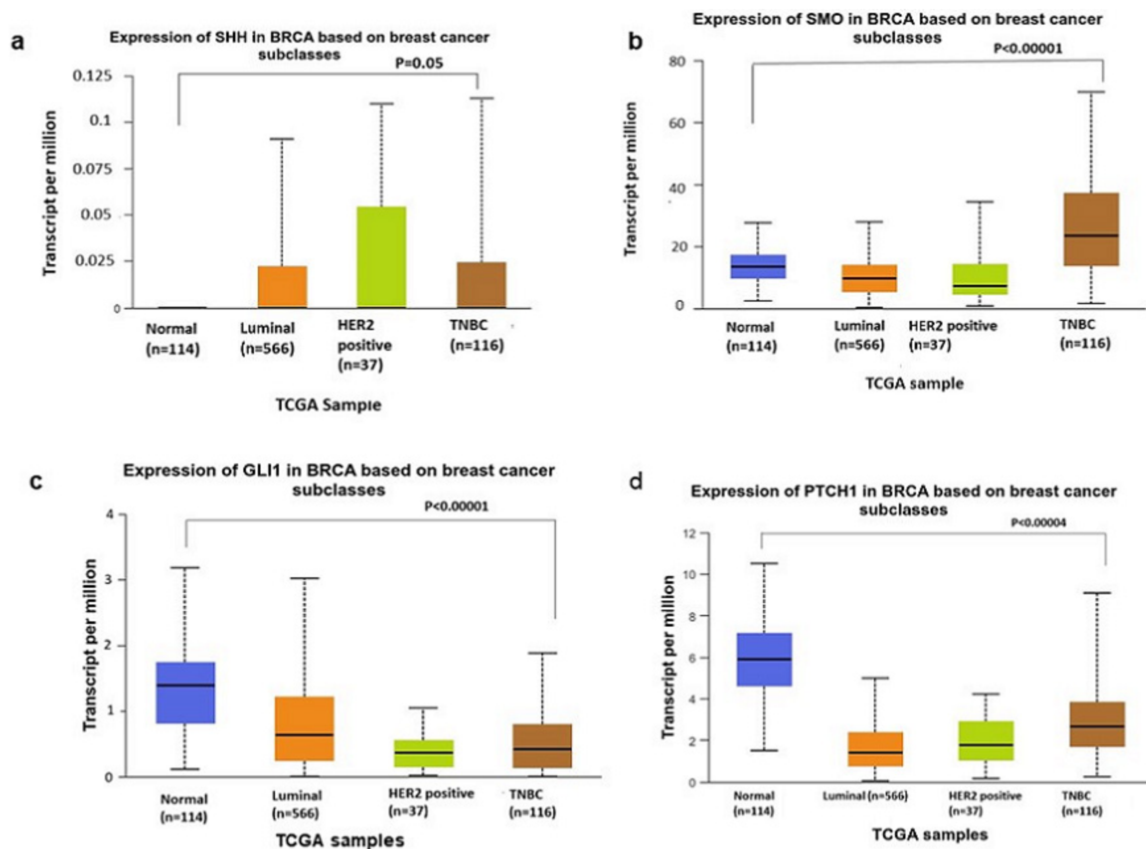

Figure 2. *SHH, SMO, PTCH1,* and *GLI1* Expression Patterns in Different Breast Cancer Subtypes in the TCGA Public Dataset. The expression pattern was evaluated based on the mRNA Z score. One-way ANOVA analysis compared genes' transcripts per million (TPM) values between normal and BC subtypes. P value < 0.05 was considered statistically significant. BRCA, Breast invasive carcinoma

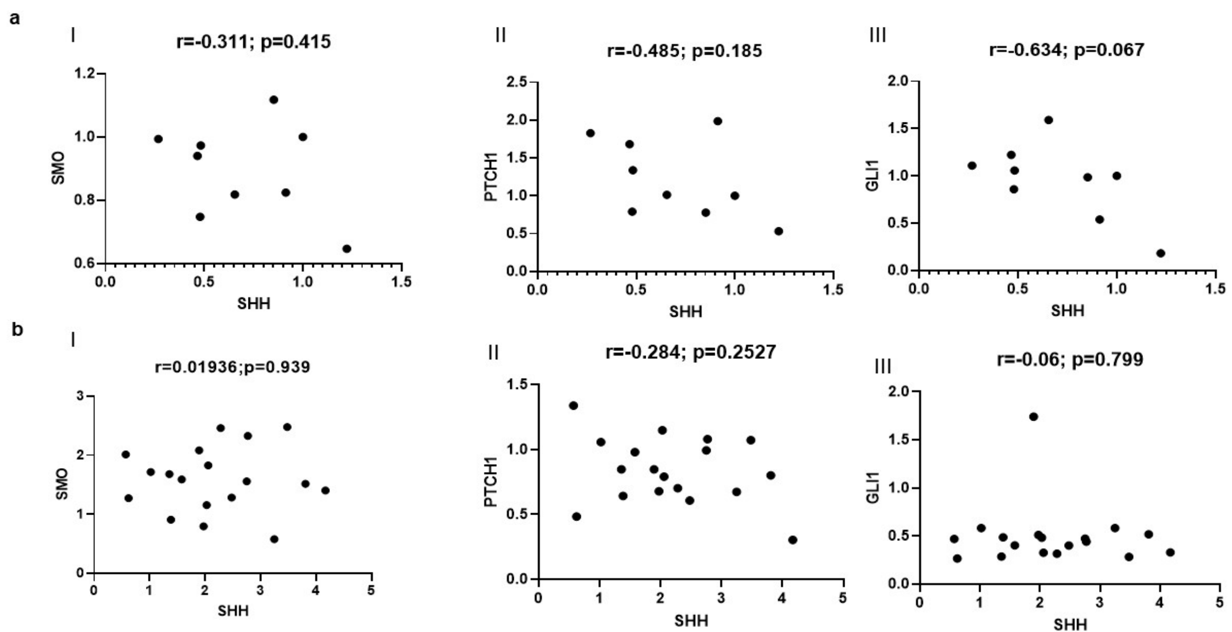

Figure 3. Correlation Analysis. Scatter plots showing the Correlation of the expression pattern between *SHH* and associated molecules *SMO, PTCH1*, and *GLI1* in normal (a) and TNBC tumor tissue (b). In this correlation analysis, p-values were determined by Pearson's correlation test.

including Luminal, Her2 positive, and TNBC. All subtypes exhibited elevated SHH expression compared to normal samples (Figure 2a). While Luminal and Her2 positive samples showed reduced SMO expression, TNBC patients showed significantly higher SMO levels than normal samples (Figure 2b). Lower *GLI1* expression was observed across all breast cancer subtypes compared to normal samples, with TNBC showing a particularly significant reduction (Figure 2c). Similarly, all subtypes exhibited decreased *PTCH1* expression relative to normal samples. Among them, the luminal subtype showed lowest *PTCH1* expression, while TNBC also showed a marked reduction (Figure 2d). Analysis of TCGA dataset revealed higher SHH expression but lower levels of SMO, *PTCH1* and *GLI1* expression in primary breast tumor tissue (Suppl Figure 1).

Correlation between the expression pattern of SHH and its associated molecules of Hh signaling pathway in normal and TNBC tumor tissue

SHH was found to be negatively correlated with SMO, *GLI1*, and *PTCH1* where SHH expression was lower and SMO, *PTCH1*, and *GLI1* expression was higher (although not significant) in normal breast tissue (Figure 3a). When we checked for correlation in tumor tissue, a negative correlation was observed for SHH, *GLI1*, and *PTCH1* where higher SHH expression was inversely correlated with *GLI1* and *PTCH1* gene expression (Figure 3b II, III). Increased SMO and SHH expression were positively correlated (Figure 3b I).

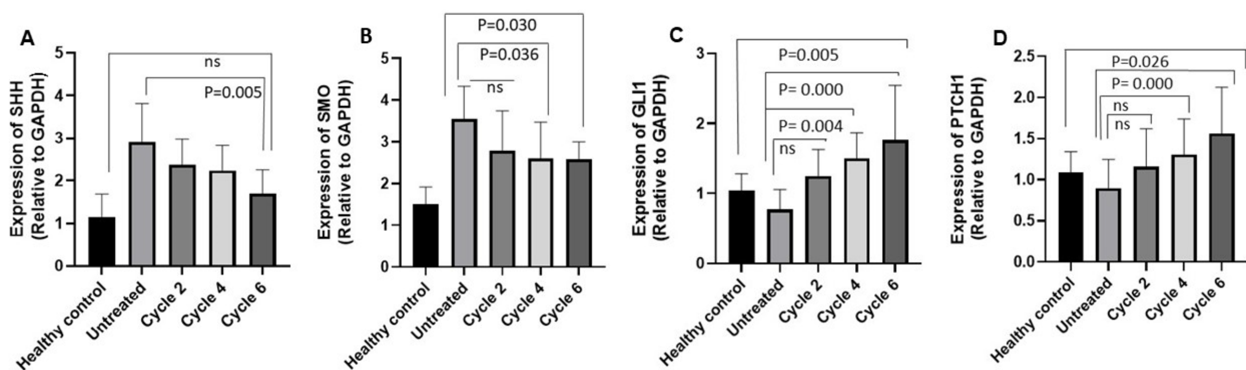

Figure 4. Expression pattern of *SHH, SMO, GLI1*, and *PTCH1* in Blood Samples of TNBC. qRT-PCR was Utilized to Determine Gene Expression Patterns from cDNAs Synthesized from Blood Samples of the Study Cohort. Normalization with *GAPDH* expression was done to calculate relative expression. The data is presented as mean ±SD. a) SHH expression pattern in TNBC subtypes after treatment with doxorubicin and cyclophosphamide. b) SMO expression pattern in TNBC subtype after treatment with doxorubicin and cyclophosphamide. c) *GLI1* expression pattern in TNBC subtypes after treatment with doxorubicin and cyclophosphamide. d) *PTCH1* expression pattern in TNBC subtypes after treatment with doxorubicin and cyclophosphamide. The number of TNBC samples was 11. Healthy control subjects were 10. P-value < 0.05 was considered statistically significant. ns=not significant.

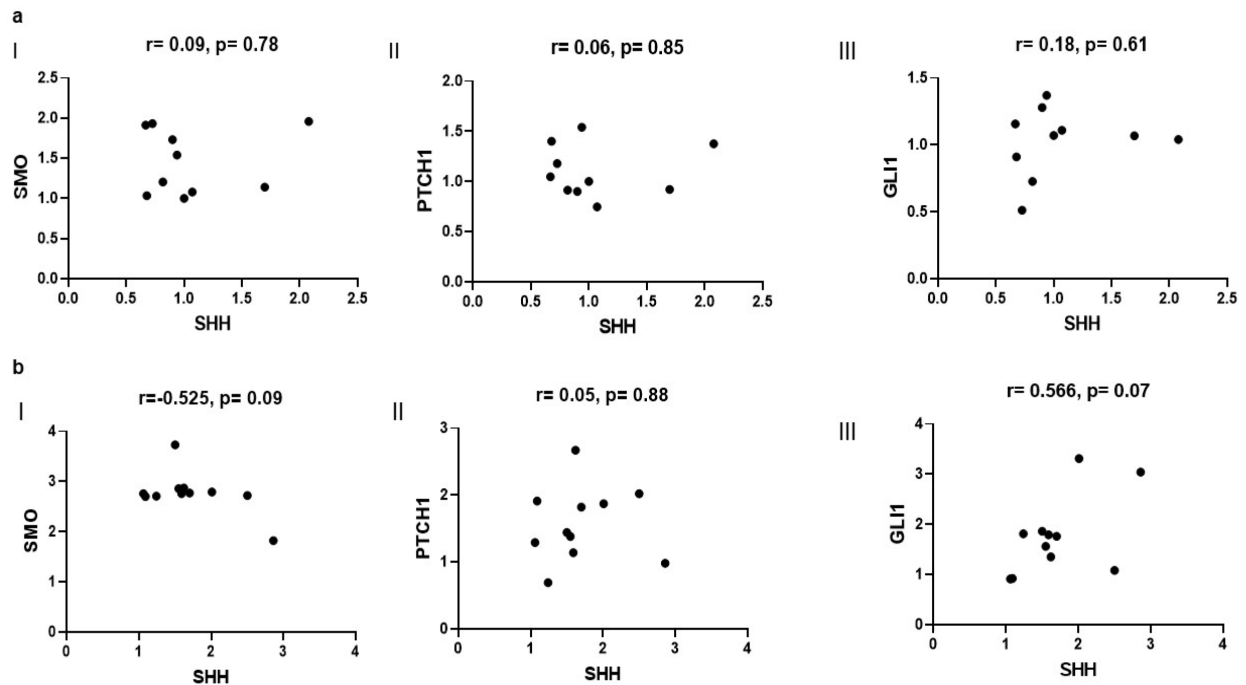

Figure 5. Correlation Analysis. Scatter plots showing the correlation of the expression pattern between Hh signaling molecule *SHH* and *SMO, GLI1,* and *PTCH1* in the healthy control sample (a) and after six chemotherapeutic cycle samples (B). In this correlation analysis, p-values were determined by Pearson's correlation test.

Expression of SHH signaling pathway genes in the TNBC subtype after treatment with doxorubicin and cyclophosphamide

We have analyzed the expression of SHH, SMO, GLI, and *PTCH1* in TNBC before and after receiving doxorubicin and cyclophosphamide chemotherapy. We have also checked the same genes in healthy control subjects. Patients received drugs every three weeks (21 days) and we evaluated gene expression at untreated conditions and after the 2nd, 4th, and 6th chemotherapeutic cycles. These two drugs decreased SHH expression sequentially after the chemotherapeutic cycle in TNBC patients. After the 6th chemotherapeutic cycle, the SHH expression was decreased significantly (p=0.005) when we compared with the untreated group. After six cycles of chemotherapy, no significant difference was observed with health control (Figure 4a). Compared to the untreated group, SMO expression was decreased sequentially after every two chemotherapeutic cycles in TNBC patients, and the mean difference in expression was significant after the 4th and 6th chemotherapeutic cycles. However, after the 6th chemotherapeutic cycle, the SMO expression was still higher compare to controls (Figure 4b). Doxorubicin and cyclophosphamide have a good impact on *GLI1* expression when checked for expression patterns after different chemotherapeutic cycles. These two drugs were found to significantly elevate *GLI1* expression after the 4th and 6th chemotherapeutic cycle in TNBC patients compared to the untreated group. Interestingly, after the 6th chemotherapeutic cycle, the *GLI1* expression was significantly (P<0.005) higher than the healthy control group as well (Figure 4c). We found a significant effect of doxorubicin and cyclophosphamide on *PTCH1* expression; these two drugs were found to elevate *PTCH1* expression after the 2nd, 4th, and 6th chemotherapeutic cycle in TNBC patients compared to the untreated group. When compared with the healthy control group, after the 6th chemotherapeutic cycle, the *PTCH1* expression was significantly higher (P<0.026) (Figure 4d).

Correlation between the expression pattern of SHH and its associated molecules of Hh signaling pathway in healthy control and 6-cycle chemotherapy-treated group

SHH was found to be marginally correlated positively with SMO, *GLI1*, and *PTCH1* in healthy control blood tissue samples (Figure 5a). When we checked for correlation of expression of SHH and its associated molecules SMO, *GLI1*, and *PTCH1* in the 6-cycle chemotherapy-treated group blood sample, a healthy control-like correlation pattern was observed for *PTCH1*, and *GLI1* showed a more favorable correlation than control. However, SHH and SMO correlated differently than control (Figure 5b).

Discussion

Breast cancer is a heterogeneous group of tumors showing different behaviors, prognosis, and response to treatment. Gene expression studies revealed several major subtypes of breast cancer. Although little data on breast cancer is available on Bangladeshi patients, clinical data on breast cancer subtypes and their treatment efficacy are limited. We, therefore, investigated the expression pattern of the Hh signaling pathway genes SHH, SMO, *GLI1*, and *PTCH1* in TNBC during chemotherapeutic treatment to identify the molecular changes related to the therapeutic outcome of breast cancer. To do this, first, we have checked the expression of Hh pathway genes in the

TCGA-BRCA dataset. TCGA dataset is a widely accepted cancer database that has more than 20000 patient data of different cancers. We have observed upregulation of SHH and SMO and downregulation of *GLI1* and *PTCH1* in breast invasive carcinoma (BRCA) patients compared to normal individuals (Suppl Figure 1). We found similar results when we checked for expression of the same gene panel in TNBC breast tumor tissue of Bangladeshi patients (Figure 1). Next, we analyzed subtype-specific expression patterns of the Hh pathway genes SHH, SMO, *GLI1*, and *PTCH1* in the TCGA dataset and found significantly decreased expression of *GLI1* and *PTCH1* and slightly increased expression of SMO and SHH in the TNBC subtype (Figure 2). The TCGA data analysis results aligned with our results on tumor and blood tissue collected from the TNBC patients (Figure 1, 4). Overexpression of SHH was also observed in our previous study [28] and other studies [32,33]. It was also found that, in breast cancer, both ductal and invasive carcinoma expressed a minute amount of *PTCH1* and low *PTCH1* expression exacerbated the physiology of the cancer patients [34]. However, in one study opposite functional expression patterns for *GLI1* and *PTCH1* are mentioned [35]. Although there is controversy regarding the role of Hh signaling pathway genes, our results are in line with the TCGA data set. An altered correlation between SHH and associated molecules was observed in tumor tissue when compared with control tissue (Figure 3). Next, we have given an interest in identifying the roles of chemotherapy, if any, in TNBC patients receiving doxorubicin and cyclophosphamide combination chemotherapy as a first-line treatment. As it is already established that combination therapy has a good impact on positive outcomes of the disease [35, 36], our focus was to evaluate the involvement of Hh signaling pathway genes in chemotherapeutic outcomes. To our surprise, very interestingly, the Hh signaling molecules SHH, SMO, *GLI1*, and *PTCH1* were altered after different chemotherapeutic cycles of doxorubicin and cyclophosphamide. In the case of SHH and SMO expression pattern, even though the expression was still higher compared to the healthy control, the level of expression was decreased after every two cycles of chemotherapeutic treatment (Figure 4 a, b). Significantly higher expression patterns of *GLI1* and *PTCH1* in TNBC patients than in the untreated group and healthy control were observed after the 6th chemotherapeutic cycle (Figure 4 c, d). Correlation data shows that, after 6 chemotherapeutic cycles, the SHH's correlation pattern with SMO, *GLI1*, and *PTCH1* was close to the results when we correlated the expression of the same genes in the healthy control group (Figure 5). These results suggest that cyclophosphamide and doxorubicin help to decrease the disease burden increasing expression of *PTCH1* and *GLI1* to normal level in TNBC. Although we have significant results regarding the correlation between the SHH signaling pathway gene expression and doxorubicin and cyclophosphamide combination treatment in TNBC, this study has some limitations too. First, we could not conclude how these drugs alter the expression pattern of the Hh signaling molecules SHH, SMO, *GLI1*, and *PTCH1*. Second, we did not follow up with the patients after the 6th chemotherapeutic cycle to know about the expression pattern and their survival after finishing the treatment. Third, our sample size was only 18 TNBC patients. Experimenting with a larger sample size could be more justified for a solid conclusion. Based on the limitations, a larger sample size along with chemotherapeutic involvement with epigenetics and/or epitranscriptomics can be checked to find out the possible role of the drugs in Hh pathway gene alteration. Moreover, follow-up data on the expression pattern could more strongly justify our result. In this research, we have focused only on the TNBC subtype because of its critical nature in treatment. Studies with other subtypes are also should be done to find out the overall involvement of doxorubicin and cyclophosphamide in breast cancer treatment strategy. Even after all the limitations, our study has unique findings. We have evaluated the correlation of expression patterns of SHH, SMO, *GLI1*, and *PTCH1* and chemotherapy in TNBC patients for the first time. The findings of this work are well justified with the TCGA dataset. Overall, we can conclude that doxorubicin and cyclophosphamide combination treatment in TNBC decreases disease burden mainly by increasing the expression of *PTCH1* and *GLI1* along with a substantial decrease in the expression of SHH and SMO. These genes can be focused for further research on targeted therapy in TNBC treatment.

Author Contribution Statement

Conceived and designed the analysis: Ramendu Parial, Shafiqul Islam, Abu Shadat Mohammod Noman, Muhammad Mosaraf Hossain. Collected the data and samples: Shafiqul Islam, Ridwan Ahmed, Nusrat Jerin, Sabbir Alam, Ali Asgar Chowdhury, Shakera Ahmed, Md. Mizanur Rahman. Contributed data generation: Shafiqul Islam, Ridwan Ahmed, Nusrat Jerin, Sabbir Alam, Md. Mizanur Rahman. Performed the analysis: Ramendu Parial, Mohammed Moinul Islam, Muhammad Mosaraf Hossain, Md. Mizanur Rahman. Wrote the paper: Ramendu Parial, Muhammad Mosaraf Hossain. Supervision: Muhammad Mosaraf Hossain, Mohammed Moinul Islam, Ramendu Parial, Abu Shadat Mohammod Noman.

Acknowledgements

General

The authors have acknowledged the patient support provided by the Department of Surgery, Chittagong Medical College, and the excellent research infrastructure of the Centre for Research Excellence, Department of Biochemistry & Molecular Biology, University of Chittagong, Chattogram, Bangladesh.

Funding statement

This work has been supported by the Research and Publication Cell (Ref No: 38/GoBe/PoRi/ProKa/Doptor/CU/2021). Ramendu Parial received research grant from the Research and Publication Cell, University of Chittagong, Chattogram, Bangladesh.

Data availability statement

The datasets generated and analyzed during the current study are not publicly available due to the policy of our laboratory but are available from the corresponding author upon reasonable request.

Ethical declaration

All procedures performed in this study involving human participants were under the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The collection and processing of the sample for this research work was performed through a collaborative approach. Ethical permission was approved by Chittagong Medical College and Hospital (CMCH). The ethical permission number of our research project is CMC/PG/2017/38. The participants were given a clear explanation of the objective of this study, and both written and verbal informed consent were obtained.

Conflict of Interests

The authors have no relevant financial or non-financial interests to disclose.

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