**Prognostic significance of hedgehog signaling network-related gene expression in breast cancer patients**

Julia Kuehn | Nancy Adriana Espinoza-Sanchez | Felipe C. O. B. Teixeira | Mauro S. G. Pavão | Ludwig Kiesel | Balázs Győrffy | Burkhard Greve | Martin Götte

1Department of Gynecology and Obstetrics, Münster University Hospital, Münster, Germany
2Department of Radiotherapy-Radiooncology, Münster University Hospital, Münster, Germany
3Instituto de Bioquímica Médica Leopoldo de Meis, Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
4Department of Bioinformatics, Semmelweis University, and Semmelweis University 2nd Department of Pediatrics, TTK Momentum Cancer Biomarker Research Group, Budapest, Hungary

**Correspondence**
Martin Götte, Department of Gynecology and Obstetrics, Münster University Hospital, Albert-Schweitzer-Campus 1, D11, 48149 Münster, Germany.
Email: mgotte@uni-muenster.de

**Funding information**
Deutscher Akademischer Austauschdienst, Grant/Award Number: 91749472; Ministry for Innovation and Technology in Hungary, Grant/Award Number: 2020-4.1.1.-TKP2020; H2020 Marie Sklodowska-Curie Actions, Grant/Award Number: 645756; Deutsche Forschungsgemeinschaft, Grant/Award Numbers: GO 1392/8-1, GR4743/5-1; Conselho Nacional de Desenvolvimento Científico e Tecnológico, Grant/Award Numbers: 290231/2017-5-SWE, 302171/2018-8-PQ

**Abstract**
Breast cancer continues to be a serious public health problem. The role of the hedgehog pathway in normal development of the mammary gland as well as in carcinogenesis and progression of breast cancer is the subject of intense investigation, revealing functional interactions with cell surface heparan sulfate. Nevertheless, its influence on breast cancer prognosis, and its relation to specific sulfation motifs in heparan sulfate have only been poorly studied in large patient cohorts. Using the public database KMplotter that includes gene expression and survival data of 3951 patients, we found that the higher expression of *SHH, HHAT, PTCH1, GLI1, GLI2*, and *GLI3* positively influences breast cancer prognosis. Stratifying patients according to the expression of hormone receptors, histological grade, lymph node metastasis, and systemic therapy, we observed that *GLI1*, *GLI2*, and *GLI3* expression, as well as co-expression of *SHH* and *ELP1* were associated with worse relapse-free survival in patients with HER2-positive tumors. Moreover, *GLI1* expression in progesterone receptor-negative tumors and *GLI3* expression in grade 3 tumors correlated with poor prognosis. *SHH*, in a panel of cell lines representing different breast cancer subtypes, and *HHAT, PTCH1, GLI1, GLI2*, and *GLI3* were mostly expressed in cell lines classified as HER2-positive and basal-like. Expression of *SHH, HHAT, GLI2*, and *GLI3* was differentially affected by...
overexpression of the heparan sulfate sulfotransferases HS2ST1 and HS3ST2 in vitro. Although high HS2ST1 expression was associated with poor prognosis in KMplotter analysis, high levels of HS3ST2 were associated with a good prognosis, except for ER-positive breast cancer. We suggest the GLI transcription factors as possible markers for the diagnosis, treatment, and prognosis of breast cancer especially in HER2-positive tumors, but also in progesterone receptor-negative and grade-3 tumors. The pathway interaction and prognostic impact of specific heparan sulfate sulfotransferases provide novel perspectives regarding a therapeutical targeting of the hedgehog pathway in breast cancer.

KEYWORDS
breast cancer, ELP1, hedgehog pathway, Heparan sulfate, KM plotter, prognosis, survival analysis

1 INTRODUCTION

During embryonic development in vertebrates, defined signaling pathways are essential for the maintenance of stem cells, their survival, differentiation, and proliferation, as well as for the regulation of the polarity of the tissues and tissue regeneration. Among those is the hedgehog (Hh) pathway. In humans, its signaling is mediated by three Hh ligands: sonic Hh (SHH), Indian Hh (IHH) and desert Hh (DHH), the receptors patched (PTCH1) and smoothened (SMO), and the transcription factors GLI1, GLI2, and GLI3. GLI1 and GLI2 are activators, while GLI3 acts both as a negative and positive regulator of the Hh pathway. However, in SHH signaling, GLI3 has a pivotal function in the regulation of the SHH pathway, working as a transcription factor. Once the Hh pathway becomes activated, GLI1 or GLI2 are phosphorylated, separate from the suppressor of fused protein (SUFU) in the cytoplasm and translocate to the nucleus to promote the transcription of genes including the stem cell-related genes NANOG and SOX2, genes involved in epithelial to mesenchymal transition (EMT) such as the Snail family transcriptional repressor 1 (SNAI1), cell cycle regulators such as Cyclin-D1 and MYC, genes involved in apoptosis including caspase-9 (CASP9) and BCL2, and genes linked to angiogenesis, such as vascular endothelial growth factor (VEGF), angiopoietin-1 and -2 (ANGPT1, 2). Notably, the activity of the Hh pathway is modulated by cell surface heparan sulfate. As with other signaling pathways, constituents of the Hh pathway can be mutated or overexpressed in cancer, participating in its induction and progression. For example, the mutation in PTCH1 causes the rare Gorlin-Goltz syndrome, where patients have a risk to develop brain, muscle, and skin tumors. Also, mutations in SMO and SUFU may result in the development of basal cell carcinoma and medulloblastoma, respectively. The overexpression of some of the Hh pathway ligands has also been observed in solid tumors, resulting in tumor development and progression as in the case of gastric, pancreas, prostate, lung, and liver cancer. Interestingly, SHH overexpression is present in the most aggressive types of breast cancers.

Breast cancer is one of the most common causes of cancer-related death among working-age women worldwide. Despite advances in diagnostic and treatment methods, much remains to be learned about the mechanisms by which this type of disease continues to be devastating. Breast cancer is a very complex disease because it is highly heterogeneous. This is reflected by the broad classification of this neoplasm regarding the expression of hormone receptors, the histology, the size of the tumor, and the presence of metastases. Several signaling pathways and epigenetic factors contribute to the progression of breast cancer. For example, we have recently shown that altered expression of the NF-kB pathway and the methyltransferase SETD3 are associated with subtype-specific poor survival of breast cancer patients. However, the role of the Hh pathway in the initiation and progression of breast cancer is not well understood. GLI2 and PTCH1 play an important role in the development of the mammary gland in murine models. Interestingly, the downregulation of PTCH1 and GLI2 causes a duc tal dysplasia that is similar to hyperplasia seen in human breast cancer affecting ductal morphogenesis. Genomic studies have demonstrated a role of some genes related to the Hh pathway in the initiation and progression of breast cancer. In another study, in 52 breast tumor specimens, high levels of nuclear GLI1 staining was observed and correlated with the expression of estrogen receptor (ER) and the histologic grade. In a cohort of 400 triple-negative breast cancer patients, the overexpression of Hh correlated with a significantly poorer overall survival (OS). The sera of 110 (45 operable and 65 with progressive metastasis) breast cancer patients and 30 healthy controls were analyzed to
detect the concentration of SHH and interleukin-6 (IL6). The authors found that SHH and IL6 were more highly expressed in patients with lymph node-positive status and progressive metastasis and correlated with worse OS. However, as the authors concluded, more studies should be carried out to corroborate these results in a larger cohort of patients and a longer follow-up study. Also, it is necessary to stratify patients for different molecular subtypes to identify whether the Hh pathway-related genes participate in the progression of breast cancer in a specific classification. Furthermore, no integrated study of the prognostic impact of the hedgehog pathway on breast cancer and its relation to heparan sulfate has been obtained so far.

Here, using the online database www.kmplot.com/breastcancer we analyzed the expression of several Hh pathway-related genes including SHH, Hedgehog Acalytransferase (HHAT), GLI1, GLI2, GLI3, and PTCH1, and correlated them with the relapse-free survival (RFS) and OS of breast cancer patients. Moreover, we evaluated the prognostic impact of two heparan sulfate sulfotransferases, HS2ST1, HS3ST2 that are functionally linked to breast cancer progression and hedgehog signaling, and of another novel modulator of SHH, elongation protein ELP1 in our patient collective. First, we showed that all genes except for GLI3 are more expressed in breast cancer samples than in normal tissues. Then, in a breast cancer cohort of 3951 patients for RFS and 1402 patients for OS, we found that all analyzed genes were associated with better RFS and that only HHAT also correlated with better OS. Stratifying the patients for different clinical types, we observed that the expression of the three GLI genes correlates with worse RFS in human epidermal growth factor receptor-positive (HER2-positive) samples. Although our results suggest some genes of the Hh pathway as good prognostic factors, the GLI (1-3) transcription factors (TF) emerge as both prognostic factors and possible therapeutic targets for patients with HER2-positive tumors. Moreover, functional in vitro assays demonstrate the regulatory interplay of the hedgehog pathway and heparan sulfate biosynthesis, and a differential impact of HS2ST1 and HS3ST2 on breast cancer prognosis, which appear more prominent in triple-negative breast cancer.

2 | MATERIALS AND METHODS

2.1 | Kaplan-Meier plots

The publically available gene expression database Kaplan-Meier plotter (KM plotter) (https://kmplot.com) was used to analyze the prognostic value of the hedgehog signaling related-genues in the relapse-free survival of breast cancer patients. The establishment of the database of the gene expression data and survival information was performed as were previously described in Espinoza-Sánchez et al., Hassan et al., and Györffy et al. Patients were stratified by ER, progesterone receptor, HER2 expression, and lymph node status, as well as for the molecular classification, and grading. We analyzed the expression of some genes of the Hh pathway and visualized their correlation to survival by generating Kaplan-Meier survival plots. The Affymetrix probe set IDs for each analyzed gene were SHH-207586_at, HHAT-219687_at, GLI1-206646_at, GLI2-228537_at, GLI3-227376_at, PTCH1-209816_at, ELP1-202490_at, HS2ST1-203283_s_at, and HS3ST2-219697_at.

2.2 | Cell culture

All human breast cancer cell lines were purchased from ATCC/LGC Promochem (Wesel, Germany). T47D, MDA-MB-453, MDA-MB-468, MDA-MB-231, and SKBR3 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (FCS), 1% glutamine, and 1% penicillin/streptomycin in a humidified atmosphere of 7.5% CO2 at 37°C. MCF-7 cells were maintained in RPMI containing 10% FCS, 1% glutamine, and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO2 at 37°C. BT474 cells were maintained in RPMI containing 20% FCS, 1% glutamine, 1% penicillin/streptomycin, and 0.01 mg/ml insulin in a humidified atmosphere of 5% CO2 at 37°C. MDA-MB-231 and MCF-7 cells stably transfected with a pcDNA3.1 control plasmid (Invitrogen) or a plasmid allowing for expression of the open reading frame (1104 bp) of human HS2ST1 (NCBI Reference Sequence: NM_012262) or human HS3ST2 (NCBI Reference Sequence: NM_006043.1) in the vector pReceiver-M02 under the control of the cytomegalovirus promoter (RZPD/ImaGenes) as previously described. For culture of stably transfected cells, media were supplemented with 600 mg/ml G418 (Gibco BRL).

2.3 | Quantitative real-time PCR

For transcriptional analysis, we obtained RNA from the cultured human breast cancer cell lines listed above using the InnupREP RNAMini Kit (Biometra) according to the manufacturer’s instructions. RNA was subsequently reverse-transcribed into cDNA using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific), random hexamer primers, and M-MuLV reverse transcriptase. The gene expression levels were measured in an ABI
PRISM 7300 Sequence Detection System (Thermo Fisher Scientific) using SYBR Green dye (Thermo Fisher Scientific) and the cycling conditions recommended by the manufacturer. Quantitative real-time PCR was conducted in triplicates for each gene of interest using the $2^{\Delta\Delta C_t}$ value. To normalize gene expression, Cycle threshold ($C_t$) values from each sample were normalized to its corresponding $\beta$-ACTIN $C_t$. Melting curve analysis was performed to confirm specific product amplification. Primer sequences were confirmed by NCBI BLAST analysis and are listed in Table S1.

### 2.4 Network analysis

To generate in silico protein interaction networks for the gene products that we analyzed in the KMplot tool, the STRING v11 (http://string-db.org/) resource was used. All interactions were predicted with a high confidence threshold of 0.700, and all active predictive methods were allowed. For the enrichment analysis, STRING implements well-known classification systems such as Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG).

### 2.5 Statistical analysis

To calculate Kaplan-Meier survival curves and the number-at-risk in the R statistical environment, we utilized the Kaplan-Meier-Plotter database via the statistical package “survival” to compute Cox proportional hazard regression and to calculate the hazard ratio (and 95% confidence intervals) and log-rank P for each gene. Kaplan-Meier plots were drawn to visualize data. In each analysis, the median expression was used as a cut-off to assign samples into high and low expression cohorts. The false discovery rate (FDR) was computed using the brainwaver library in R. In the case of qPCR results, the Prism software version 6.0 (GraphPad) was used for statistical analysis. Unpaired Student’s $t$-test and one-way analysis of variance (ANOVA) test with Tukey as a post hoc test were applied. Significant p values are indicated as follows: *$p \leq .05$; **$p \leq .01$; ***$p \leq .001$.

### 3 RESULTS

#### 3.1 The expression of Hh pathway-related genes differs between normal and cancer tissue samples and is associated with a better relapse-free survival

To investigate the role of Hh pathway related-genes in breast cancer progression, we compared the expression levels of $SHH$, $HHAT$, $GLI1$, $GLI2$, $GLI3$, and $PTCH1$ between normal and breast cancer tissues samples. We found that all of the analyzed genes except for $GLI3$ are more highly expressed in the tumor samples (Table 1). Subsequently, in a collective of 3951 patients for RFS, and 1402 patients for OS, we analyzed the impact of the expression of genes related to the Hh pathway on the prognosis of breast cancer patients. Our results show that the expression of $SHH$, $HHAT$, $GLI1$, $GLI2$, $GLI3$, and $PTCH1$ correlates with better RFS but only the expression of $HHAT$ was also associated with

| Gene | Affymetrix ID | Tissue | Number of samples | Median expression | $p$ value |
|------|---------------|--------|------------------|------------------|-----------|
| $SHH$ | 207586_at | Normal | 76 | 37 | .0063* |
|       |               | Tumor  | 6547 | 45 |          |
| $HHAT$ | 219687_at | Normal | 76 | 214 | .00189* |
|       | Measured | Tumor  | 6547 | 278 |          |
| $GLI1$ | 206646_at | Normal | 76 | 61 | .0114* |
|       | Measured | Tumor  | 6547 | 80 |          |
| $GLI2$ | 228537_at | Normal | 76 | 221 | .038* |
|       | Measured | Tumor  | 6547 | 189 |          |
| $GLI3$ | 227376_at | Normal | 76 | 856 | .0711 |
|       | Measured | Tumor  | 6547 | 1034 |          |
| $PTCH1$ | 209816_at | Normal | 76 | 37 | .00002* |
|       | Measured | Tumor  | 6547 | 59 |          |

*p value considered significant in the median expression of the respective gene in normal tissue versus cancer sample tissue after Benjamini-Hochberg (FDR, below 10%) correction for multiple testing.

#### TABLE 1 Expression of hedgehog signaling network-related genes at the mRNA level in normal tissue versus cancer tissue samples
Figure 1  Prognostic significance of hedgehog signaling network-related gene expression for relapse-free survival in breast cancer patients. The patient cohort was divided into groups of low (black) and high (red) gene expression according to respective median gene expression. Patients were censored at the threshold. Survival is displayed as Kaplan-Meier curves; computed HR, 95% CI, and log-rank p values are given. A, SHH (207586_at, n = 3951); B, HHAT (219687_at, n = 3951); C, GLI1 (206646_at, n = 3951); D, GLI2 (228537_at, n = 1764); E, GLI3 (227376_at, n = 1764); F, PTCH1 (209816_at, n = 3951)
better OS (Figure 1 and Table 2). The clinicopathological characteristics of the patients investigated in the present study were previously described in Espinoza-Sánchez et al., and are shown in Table S2.

### 3.2 Expression of GLI1 correlates with poor prognosis in patients with progesterone receptor-negative tumors, while SHH, HHAT, GLI1, GLI2, GI3, and PTCH1 are associated with good prognosis in Luminal tumors

The classification of the patients according to the expression of hormone receptors is pivotal for treatment decisions and the prognosis of the patients with breast cancer. To investigate if the Hh pathway-related genes play a specific role in a given classification, we stratified the patients for the expression of ER, PR, and HER2. Table 3 shows that, when patients have ER tumors that express only HHAT (HR = 0.77; p = .0019) they have a good prognosis. We found no association with the expression of the genes and the RFS in ER- tumors. In PR tumors only HHAT (HR = 0.62; p = .0069) was associated with better RFS. In contrast, in PR-tumors the expression of GLI1 (HR = 1.4; p = .026) correlates with worse RFS (Table 3). In the Luminal molecular classification, which is based on the expression of ER and PR, we found that the expression of SHH, HHAT, GLI2, and PTCH1 correspond to a better RFS in both Luminal A and B tumors. On the other hand, the expression of GLI1 (HR = 0.69; p = .000023) and GLI3 (HR = 0.65; p = .00071) were associated with good prognosis only in patients of the Luminal A classification (Table 3). Interestingly, in basal-like breast cancer, which has the worst outcome in patients, the expression of SHH (HR = 0.74; p = .018), GLI2 (HR = 0.54; p = .00019), and PTCH1 (HR = 0.67; p = .0023) correlates with good prognosis (Table 3).

### 3.3 The GLI (1-3) transcription factors influence the prognosis of breast cancer patients with HER2-positive tumors

Overexpression of HER2 can be found in approximately in a range of 20%-30% of the tumors of breast cancer patients. Among the different types of breast cancer, it is one of the most aggressive tumor characterized by higher recurrence rates, and shortened survival. For this reason, we decided to analyze the expression of SHH, HHAT, GLI1, GLI2, GI3, and PTCH1 in patients with HER2-positive and -negative tumors and analyzed the correlation of the gene expression data with the patients survival. Interestingly, the high expression of the three GLI transcription factors correlates with worse RFS in patients with HER2+ tumors (GLI1 HR = 1.92; p = .0038, GLI2 HR = 1.89; p = .023, and GLI3 HR = 1.83; p = .031) (Figure 2 left panel and Table 4). In HER2- tumors the high expression of GLI2 (HR = 0.58; p = .00044) and GLI3 (HR = 0.66; p = .0071) as well as HHAT (HR = 0.5; p = .00000023), were associated with better RFS (Figure 2 right panel and Table 4). The other genes had no association with RFS (Table 4).

**Table 2** Prognostic significance of hedgehog signaling network-related gene expression for relapse-free survival and overall survival in breast cancer patients.

| Gene     | Affymetrix ID | Survival | Cases | HR (95% CI) | log-rank p value |
|----------|---------------|----------|-------|-------------|-----------------|
| SHH      | 207586_at     | RFS      | 3951  | 0.74 (0.66–0.82) | 4.2e – 8*       |
|          |               | OS       | 1402  | 0.99 (0.8 – 1.23) | 0.95           |
| HHAT     | 219687_at     | RFS      | 3951  | 0.65 (0.58 – 0.72) | 7.3e – 15*      |
|          |               | OS       | 1402  | 0.75 (0.6 – 0.93)   | 0.0075*        |
| GLI1     | 206646_at     | RFS      | 76    | 0.79 (0.71 – 0.88)   | 1.6e – 5*       |
|          |               | OS       | 6547  | 0.81 (0.66 – 1.01)   | 0.057          |
| GLI2     | 228537_at     | RFS      | 76    | 0.58 (0.5 – 0.68)    | 1.2e – 11*      |
|          |               | OS       | 6547  | 0.91 (0.66 – 1.24)   | 0.54           |
| GLI3     | 227376_at     | RFS      | 76    | 0.76 (0.65 – 0.89)   | 0.00056*       |
|          |               | OS       | 6547  | 0.83 (0.61 – 1.14)   | 0.24           |
| PTCH1    | 209816_at     | RFS      | 76    | 0.78 (0.69 – 0.86)   | 4.5e – 6*       |
|          |               | OS       | 6547  | 0.93 (0.75 – 1.15)   | 0.48           |

Abbreviations: CI, confidence interval; HR, hazard ratio; RFS, relapse-free survival; OS, overall survival. *p value considered significant in the median expression of the respective gene after Benjamini-Hochberg (FDR, below 10%) correction for multiple testing.
| Gene | Affymetrix ID | Molecular classification | Cases | HR (95% CI) | log-rank p value |
|------|--------------|--------------------------|-------|-------------|-----------------|
| SHH  | 207586_at    | ER+                      | 2061  | 0.95 (0.81–1.12) | .55             |
|      |              | ER−                      | 801   | 0.96 (0.77–1.21) | .75             |
|      |              | PR+                      | 589   | 0.99 (0.7–1.4)   | .96             |
|      |              | PR−                      | 549   | 1.03 (0.77–1.28) | .85             |
|      |              | Luminal A                | 1933  | 0.77 (0.65–0.91) | .0021*          |
|      |              | Luminal B                | 1149  | 0.79 (0.65–0.96) | .018*           |
|      |              | BLBC                     | 618   | 0.74 (0.57–0.95) | .018*           |
| HHAT | 219687_at    | ER+                      | 2061  | 0.96 (0.77–1.21) | .75             |
|      |              | ER−                      | 801   | 1.13 (0.9–1.41)  | .3              |
|      |              | PR+                      | 589   | 0.62 (0.43–0.88) | .0069*          |
|      |              | PR−                      | 549   | 1.14 (0.85–1.52) | .39             |
|      |              | Luminal A                | 1933  | 0.64 (0.54–0.77) | .00000055*      |
|      |              | Luminal B                | 1149  | 0.81 (0.67–0.98) | .029*           |
|      |              | BLBC                     | 618   | 1.03 (0.8–1.33)  | .81             |
| GLI1 | 206646_at    | ER+                      | 2061  | 1 (0.85–1.18)    | .97             |
|      |              | ER−                      | 801   | 1.22 (0.97–1.53) | .082            |
|      |              | PR+                      | 589   | 1.07 (0.76–1.52) | .69             |
|      |              | PR−                      | 549   | 1.4 (1.04–1.88)  | .026*           |
|      |              | Luminal A                | 1933  | 0.69 (0.58–0.82) | .000023*        |
|      |              | Luminal B                | 1149  | 1.05 (0.87–1.27) | .63             |
|      |              | BLBC                     | 618   | 0.83 (0.65–1.07) | .16             |
| GLI2 | 228537_at    | ER+                      | 762   | 0.74 (0.55–1)    | .048            |
|      |              | ER−                      | 347   | 0.9 (0.65–1.25)  | .53             |
|      |              | PR+                      | 489   | 0.73 (0.5–1.08)  | .12             |
|      |              | PR−                      | 372   | 0.83 (0.58–1.18) | .29             |
|      |              | Luminal A                | 841   | 0.54 (0.42–0.7)  | .0000016*       |
|      |              | Luminal B                | 407   | 0.66 (0.49–0.91) | .0992*          |
|      |              | BLBC                     | 360   | 0.54 (0.39–0.75) | .00019*         |
| GLI3 | 227376_at    | ER+                      | 762   | 0.8 (0.6–1.07)   | .13             |
|      |              | ER−                      | 347   | 1.13 (0.81–1.58) | .46             |
|      |              | PR+                      | 489   | 0.81 (0.56–1.19) | .28             |
|      |              | PR−                      | 372   | 1.32 (0.92–1.9)  | .12             |
|      |              | Luminal A                | 841   | 0.65 (0.51–0.84) | .00071*         |
|      |              | Luminal B                | 407   | 0.97 (0.72–1.32) | .86             |
|      |              | BLBC                     | 360   | 1.28 (0.92–1.77) | .14             |
| PTCH1| 209816_at    | ER+                      | 2061  | 1.05 (0.89–1.24) | .53             |
|      |              | ER−                      | 801   | 1.04 (0.83–1.3)  | .72             |
|      |              | PR+                      | 589   | 0.78 (0.55–1.11) | .17             |
|      |              | PR−                      | 549   | 1.11 (0.83–1.49) | .48             |
|      |              | Luminal A                | 1933  | 0.83 (0.7–0.99)  | .035*           |
|      |              | Luminal B                | 1149  | 0.74 (0.61–0.89) | .0017*          |
|      |              | BLBC                     | 618   | 0.67 (0.52–0.87) | .0023*          |

Abbreviations: BLBC, basal-like breast cancer; CI, confidence interval; ER+, or ER−, Estrogen-receptor positive or negative; PR+, or PR−, progesterone receptor-positive or negative; HR, hazard ratio.

*p value considered significant in the median expression of the respective gene after Benjamini-Hochberg (FDR, below 10%) correction for multiple testing.
FIGURE 2  The prognostic value of the expression of the GLI transcriptions factors based on HER2 status. Kaplan-Meier relapse-free survival curves are plotted for breast cancer patients with HER2⁺ (n = 252) and HER2⁻ (n = 800), and the expression of A, GLI1; B, GLI2; and C, GLI3. Log-rank p values and hazard ratios (HRs; 95% confidence interval in parentheses) are shown.
### Table 4

| Gene      | Affymetrix ID | HER2 Status | Cases | HR (95% CI) | log-rank p value |
|-----------|---------------|-------------|-------|-------------|------------------|
| SHH       | 207586_at     | Positive    | 252   | 1.45 (0.93–2.24) | .096             |
|           |               | Negative    | 800   | 0.78 (0.6–1.01)  | .063             |
| HHAT      | 219687_at     | Positive    | 252   | 0.97 (0.63–1.49) | .89              |
|           |               | Negative    | 800   | 0.5 (0.38–0.65)  | .00000023*       |
| GLI1      | 206646_at     | Positive    | 252   | 1.92 (1.22–3.01) | .0038*           |
|           |               | Negative    | 800   | 0.8 (0.62–1.04)  | .094             |
| GLI2      | 228537_at     | Positive    | 150   | 1.89 (1.08–3.29) | .023*            |
|           |               | Negative    | 635   | 0.58 (0.43–0.79) | .00044*          |
| GLI3      | 227376_at     | Positive    | 150   | 1.83 (1.05–3.19) | .031*            |
|           |               | Negative    | 635   | 0.66 (0.49–0.9)  | .0071*           |
| PTCH1     | 209816_at     | Positive    | 252   | 0.88 (0.57–1.37) | .57              |
|           |               | Negative    | 800   | 0.87 (0.67–1.14) | .31              |

Abbreviations: CI, confidence interval; HR, hazard ratio.

*p value considered significant in the median expression of the respective gene after Benjamini-Hochberg (FDR, below 10%) correction for multiple testing.

3.4 In patients with grade 3 tumors, the expression of GLI3 is associated with worse relapse-free survival, while other Hh pathway members correlate with better relapse-free survival of patients undergoing a systemic therapy

We next determined the association of the Hh pathway related-genes with lymph nodal status and grades 1–3 analyzing a total of 1133 breast cancer patients with lymph node (LN) positive, 2020 with lymph node-negative status, 345 grade 1, 901 grade 2, and 903 grade 3 for RFS (Table S3). Even though the HR of most genes is close to or greater than 1, we did not find significant differences in the p-value between the patients who have high or low expression of the analyzed genes and independent of their LN status or grade. However, we found that the expression of GLI3 (HR = 1.59; p = .004) was associated with worse RFS in patients with grade 3 tumors. HHAT correlates with worse RFS in patients with both LN-positive (HR = 0.65; p = .000016) and LN-negative (HR = 0.7; p = .000027) tumors. Moreover, the expression of GLI (HR = 0.74; p = .0027) and GLI2 (HR = 0.67; p = .0018) were associated with good prognosis in patients with LN-positive tumors (Table S2).

It is well known that a significant percentage of breast cancer patients are resistant to commonly used therapies and part of this is due to the expression of multidrug resistance genes. In our study, we found that the expression of HHAT contributes to the good prognosis of breast cancer patients regardless of whether or not they underwent any therapeutic regimen (Table S3). SHH (HR = 0.62; p = 1.1e−09), GLI1 (HR = 0.66; p = .0000025), GLI2 (HR = 0.48; p = 2.9e−11), GLI3 (HR = 0.71; p = .0014), and PTCH1 (HR = 0.54; p = 1.4e−14) were associated with better RFS in patients that undergo therapy (Supplementary Table 4).

3.5 The prognostic value of Heparan sulfate 2-O-sulfotransferase 1 and Heparan sulfate-glucosamine 3-sulfotransferase 2 (HS2ST1 and HS3ST2) in breast cancer

Activation of the hedgehog signaling pathway is modulated by cell surface heparan sulfate (HS), a long, unbranched polysaccharide composed of repeating disaccharide units of alternating glucuronic or iduronic acid and N-acetylg glucosamine. HS undergoes sequential enzymatic modifications necessary for its proper functioning that includes deacetylation, epimerization and, sulfation by the enzymes N-deacetylases/N-sulfotransferases (NDSTs) and 2-O, 6-O, and 3-O sulfotransferases (HS2ST, HS6ST, and HS3ST, respectively). We previously showed that HS2ST1 and HS3ST2 play a very important role in the maintenance and invasiveness of breast cancer stem cells (CSCs). Also, we observed important changes in the expression patterns of pathways associated with stemness, including the Wnt and Notch pathway. Therefore, we were interested in looking for the impact of the expression of the enzymes HS2ST1 and HS3ST2 on the breast cancer patient prognosis. We found that the expression of HS2ST1 correlates with worse RFS in all the patients without any
classification (HR = 1.31; \( p = 1.4e - 06 \)), in ER-positive and ER-negative tumors (HR = 1.17; \( p = 0.018 \) and HR = 1.3; \( p = 0.15 \), respectively), as well as in Basal tumors (HR = 1.47; \( p = 0.089 \)) (Figure 3A and Table 5). Although a high HR was obtained in the patients with HER2-positive and -negative, Luminal A and B tumors, we did not observe significant differences between the patients who expressed high or low HS2ST1 levels (Figure 3A and Table 5). In contrast, the expression of HS3ST2 was associated with a better RFS in all of the patients (HR = 0.84; \( p = 0.0016 \)) and in ER-negative (HR = 0.65; \( p = 6.7e - 05 \)), HER2 (HR = 0.55; \( p = 0.0022 \)), and Basal (HR = 0.62; \( p = 0.0025 \)) tumors (Figure 3B and Table 5).

As we have previously observed that HS2ST1 and HS3ST2 influence the expression of genes associated with the stemness-Notch and Wnt pathways,31,32,42 we analyzed the co-expression of HS2ST1 and HS3ST2 with the hedgehog pathway constituents SHH, HHAT, PTCH1, GLI1, GLI2, and GLI3 and correlated them with patient. We observed that the co-expression of HS2ST1 with SHH was associated with poor RFS only in the patients without any classification by Kmplotter (HR = 1.17; \( p = 0.0042 \)), but not in subclassifications (Table S5). HS2ST1/HHAT co-expression was associated with better survival in all patients, ER-positive, HER2-negative and Luminal A tumors (HR = 0.83; \( p = 0.0069 \), HR = 0.85; \( p = 0.01 \), HR = 0.71; \( p = 0.11 \), (HR = 0.73; \( p = 0.00041 \), respectively) (Table S5). Co-expression of HS2ST1/PTCH1 had no significant association with RFS (Table S5). Interestingly, we observed that the three GLI 1 and GLI3 factors in co-expression with HS2ST1 were associated with worse survival in HER2-positive tumors, while GLI1/HS2ST1 co-expression also correlated with worse RFS in ER-negative and PR-negative tumors (Table S5). GLI3 co-expressed with HS2ST1 was associated with better RFS in HER2-negative tumors. On the other hand, GLI2 and GLI3/HS2ST1 co-expression resulted in a good prognosis in all patients, ER-positive, and Luminal A tumors (Table S5). In Table S6, we show the impact of the Hh related genes/HS3ST2 co-expression on the survival of the patients. SHH, HHAT, PTCH1, GLI1, GLI2, and GLI3/HS3ST2 co-expression have in common that they were associated with good RFS in not stratified patients, ER-negative, Luminal A, and Basal tumors (with except GLI3 had no impact on ER-negative and basal tumors). In the patients with ER-positive and HER2-negative tumors, the co-expression of HHAT, GLII, GLI2, and GLI3/HS3ST2 was associated with better survival (Table S6). HHAT and PTCH1/HS3ST2 co-expression in Luminal B tumors, while PTCH1 and HS3ST2 co-expression in HER2 tumors improved the survival of the patients (Table S6). Interestingly, GLI2 and GLI3/HS3ST2 were again associated with poor RFS in HER2-positive tumors (Table S6).

### 3.6 The expression of elongation protein 1 (ELP1) and its co-expression with SHH improves the prognosis of breast cancer patients with Luminal B, basal-like, and HER2-negative tumors

Elongator complex is composed of two copies of six subunits (ELP1-6) that are implicated in transcriptional regulation, tRNA modification, and tubulin acetylation. It has been demonstrated that a failure in the function of these proteins is associated with different diseases including cancer.43 In a recent study, it was shown that 14% of patients with SHH-expressing medulloblastoma also present loss-of-function variants across elongation protein 1 (ELP1). The authors also found that in these pediatric patients, ELP1 boosts the genetic predisposition to develop tumors together with the activation of the SHH pathway.33 We therefore wanted to investigate a possible prognostic value of ELP1 in breast cancer patients. First, we evaluated the expression of ELP1 for RFS and OS in the patients without any classification by Kmplotter analysis. We found that ELP1 correlates with better RFS (HR = 0.8; \( p = 6.7e - 05 \)), but we did not find a statistical significance for OS (HR = 0.83; \( p = 0.079 \)) (Table S7). When the patients were stratified, only in the categories LB (HR = 0.73; \( p = 0.013 \)), basal-like (BLBC) (HR = 0.63; \( p = 0.0034 \)), and HER2-negative (HR = 0.69; \( p = 0.0054 \)) the expression of ELP1 were associated with good outcome in the patients (Table S7). The other clinical statuses had no association with RFS (Table S7).

Taking into account that the synergism between SHH and ELP1 has a strong impact on the prognosis of pediatric medulloblastoma, we evaluated the impact of the co-expression of SHH and ELP1 on the prognosis of patients with breast cancer. Interestingly, just as we observed that only the expression of ELP1 was associated with better survival in LB, BLCB, and HER2-negative tumors, the co-expression of SHH/ELP1 was also associated with a good prognosis in the same classifications in addition to Luminal A tumors (Table S8).

### 3.7 Hedgehog pathway-related genes are more highly expressed in aggressive breast cancer cell lines

As transcriptomic gene expression data derived from tumor tissue are comprised of the expression signature of tumor cells and cells of the tumor stroma, we evaluated Hedgehog pathway gene expression in established breast cancer cell lines to determine tumor cell-autonomous gene expression patterns. Using quantitative real-time PCR, we studied the expression of SHH, HHAT, PTCH1,
FIGURE 3  The prognostic value of the expression of HS2ST1 and HS3ST2 in breast cancer. Kaplan-Meier relapse-free survival curves are plotted for breast cancer patients and the expression of (A) HS2ST1 and (B) HS3ST2. Data are shown for the whole patient collective (ALL) and selected molecular classifications of breast cancer. Log-rank p values and hazard ratios (HRs; 95% confidence interval in parentheses) are shown.
**GLI1, GLI2, and GLI3** in representative breast cancer cell lines of the following subtypes: Luminal, MCF-7 and T47D; Her2, BT474, and SKBR3, and Basal-like, MDA-MB-453, MDA-MB-468, and MDA-MB-231. The six analyzed genes were expressed in all cell lines. SHH was more highly expressed in the HER2+ BT474 and the basal MDA-MB-231 cell line, while it was less expressed in the Luminal cell lines T47D and MCF-7 and the basal cell line MDA-MB-468 (Figure 4A). HHAT was expressed almost at the same level in all cell lines but was most highly expressed in the HER2+ BT474 cell line and the basal-like MDA-MB-453 and MDA-MB-231 cell lines (Figure 4B). PTCH1 was expressed at a lower level in Luminal MCF-7 cells compared to the HER2+ BT474 and SK-BR3 cell lines, whereas Luminal T47D and MDA-MB-231 cells expressed PTCH1 the most (Figure 4C). In the case of GLI TFs, GLI1 and GLI2 were mainly expressed by the basal cell lines, GLI1 in MDA-MB-453 and MDA-MB-468, and GLI2 in MDA-MB-231 (Figure 4D,E). Finally, GLI3 was more highly expressed in the basal cell line MDA-MB-453, and less expressed in the Luminal MCF7 cell line while in the rest of the cell lines, it was expressed almost at the same level (Figure 4F). Notably, our results suggest that although all of the cell lines expressed the genes analyzed, they were more highly expressed in the most aggressive HER2+ and basal-like subtypes (Figure 4A–F).

### 3.8 HS2ST1 and HS3ST2 overexpression in MCF-7 and MDA-MB-231 cells affects the expression of selected Hedgehog pathway-related genes

To study the impact of HS2ST1 and HS3ST2 on the expression of the genes associated with the Hedgehog pathway, we employed the breast cancer cell lines MCF-7 and MDA-MB-231 stably overexpressing the HS2ST1 and HS3ST2 enzymes. Subsequently, we analyzed the expression of SHH, HHAT, PTCH1, GLI1, GLI2, and GLI3 by qPCR. In the Luminal A MCF-7 cells, the upregulation of both HS2ST and HS3ST2 led to the downregulation of HHAT and upregulation of PTCH1 (Figure 5A). On the other hand, in the triple-negative MDA-MB-231 cell line the overexpression of both sulfotransferases significantly increased the expression of GLI2. Moreover, MDA-MB-231

---

**Table 5** Prognostic significance of HS2ST1 and HS3ST2 expression for relapse-free survival in breast cancer patients depending on molecular classification

| Gene     | Affymetrix ID | Molecular classification | Cases | HR (95% CI) | log-rank p value |
|----------|---------------|--------------------------|-------|-------------|------------------|
| HS2ST1   | 203283_S_at   | ALL                      | 3951  | 1.31 (1.17-1.46) | 1.4e-06*         |
|          |               | ER+                      | 3082  | 1.17 (1.03-1.32) | .018*            |
|          |               | ER-                      | 869   | 1.3 (1.05-1.6)  | .015*            |
|          |               | PR+                      | 589   | 0.92 (0.65-1.3) | .62              |
|          |               | PR-                      | 549   | 1.26 (0.94-1.69) | .11              |
|          |               | HER2+                    | 252   | 1.36 (0.88-2.1) | .17              |
|          |               | HER2-                    | 800   | 1.31 (1.01-1.7) | .043             |
|          |               | Luminal A                | 1933  | 1.16 (0.98-1.38) | .082             |
|          |               | Luminal B                | 1149  | 1.19 (0.98-1.44) | .076             |
|          |               | HER2                     | 251   | 1.15 (0.78-1.69) | .47              |
|          |               | BLBC                     | 618   | 1.47 (1.14-1.89) | .003*            |
| HS3ST2   | 219697_at     | ALL                      | 3951  | 0.84 (0.75-0.94) | .0016*           |
|          |               | ER+                      | 3082  | 0.94 (0.83-1.06) | .32              |
|          |               | ER-                      | 869   | 0.65 (0.53-0.81) | 6.7e-05*         |
|          |               | PR+                      | 589   | 1.15 (0.81-1.63) | .42              |
|          |               | PR-                      | 549   | 0.99 (0.74-1.32) | .93              |
|          |               | HER2+                    | 252   | 0.89 (0.58-1.38) | .62              |
|          |               | HER2-                    | 800   | 0.95 (0.73-1.23) | .68              |
|          |               | Luminal A                | 1933  | 0.9 (0.76-1.07)  | .23              |
|          |               | Luminal B                | 1149  | 0.82 (0.68-1)    | .044             |
|          |               | HER2                     | 251   | 0.55 (0.37-0.81) | .0022*           |
|          |               | BLBC                     | 618   | 0.62 (0.48-0.8)  | .00025*          |

Abbreviations: CI, confidence interval; HR, hazard ratio.

*p value considered significant in the median expression of the respective gene after Benjamini-Hochberg (FDR, below 10%) correction for multiple testing.
cells overexpressing the HS2ST1 sulfotransferase significantly downregulated the expression of SHH and GLI3, whereas MDA-MB-231 cells overexpressing the HS3ST2 sulfotransferase significantly downregulated PTCH1 and upregulated GLI3 expression (Figure 5B). Overall, these data indicate a subtype-specific regulatory impact of heparan sulfate modifications on the expression of the hedgehog pathway in breast cancer.

3.9 | The genes of the Hedgehog pathway are associated with cancer-related molecular pathways

Even though the Hedgehog pathway is well described, all the interaction networks that this pathway shares with other proteins are not yet completely discovered. Using the String online tool, we analyzed the interaction

**FIGURE 4** Relative mRNA expression of sonic hedgehog-pathway-associated genes in breast cancer cell lines. Expression of the genes SHH (A), HHAT (B), PTCH1 (C), GLI1 (D), GLI2 (E), and GLI3 (F), was quantified by qRT-PCR in seven breast cancer cell lines representative of distinct breast cancer subtypes (luminal, basal and Her2-positive subtype). Gene expression was normalized to the expression of b-actin and is given as 10000-fold 2-deltaCt. Error bars indicate the mean and SD of individual experiments in triplicates. To test for the difference between mean gene expressions in the different breast cancer cell lines, one-way ANOVA was employed, followed by the Tukey test to correct for multiple comparisons. ANOVA, analysis of variance; mRNA, messenger RNA; qRT-PC, quantitative reverse-transcription polymerase chain reaction. *p < .05, **p < .01, ***p < .001

**FIGURE 5** Relative messenger RNA (mRNA) expression of sonic hedgehog-pathway-associated genes in breast cancer cell lines overexpressing HS2ST1 and HS3ST2. (A) MCF-7 and (B) MDA-MB-231 cells were stably transfected to overexpress HS2ST1 and HS3ST2 and then, the expression of the genes SHH, HHAT, PTCH1, GLI1, GLI2, and GLI3 were analyzed. Gene expression data were compared with the corresponding cell type stably transfected with an insertless control vector. Gene expression was normalized to the expression of b-actin the fold change was presented. Error bars indicate the mean and SD of individual experiments in triplicates. To test for the difference between mean gene expressions in the different breast cancer cell lines, unpaired Student’s t-test was employed *p < .05, **p < .01, ***p < .001
network, molecular function, cellular component, the molecular process, and KEGG pathways associated with SHH, HHAT, PTCH1, GLI1, GLI2, GLI3, PTCH1, HS2ST1, HS3ST2, and ELP1. Figure 6A shows the network of these proteins in which we can distinguish three modules. On the one hand, all the Hh-related proteins form one module and are highly interconnected. Interestingly, the second module is formed by the sulfotransferases HS2ST1 and HS3ST2, which are interconnected to the Hedgehog module through glypican-3 (GPC-3), a cell surface heparan sulfate proteoglycan with a possible tumor suppressor role in breast cancer.45,46 The third module is composed of ELP proteins that are linked to neither of the other two modules. To find the molecular function, physical interaction network, and KEGG pathway analysis, all the adjusted statistically significant values of the terms were negative 10-base log-transformed.

**FIGURE 6** Protein interaction network of SHH, HHAT, PTCH1, GLI1, GLI2, GLI3, PTCH1, HS2ST1, HS3ST2, and ELP1. A, Functional and physical interactors of the Hedgehog-related proteins obtained from http://string-db.org/. B, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. All the adjusted statistically significant values of the terms were negative 10-base log-transformed.
function, cellular component, and biological process related to the Hh pathway we used the GO enrichment analysis. Table S9 lists the 10 most significantly enriched terms (p < .05) in each category. For example, in the molecular function category, we can observe patched binding, hedgehog protein family binding, phosphorylase kinase regulatory activity, heparan sulfate sulfotransferase activity, signaling receptor binding, and ion binding. The cellular component category includes the terms, an elongator holoenzyme complex, ciliary part, and base, transcription factor elongation factor complex, plasma membrane-bounded cell projection part, and endocytic vesicle membrane. Finally, in the biological process category, the Hh proteins were associated with the smoothed signaling pathway, cell fate specification, pattern specification process, epithelial morphogenesis, and regionalization. The KEGG analysis demonstrated that the Hh-related proteins were linked to different pathways associated with cancer, including proteoglycans, cAMP, and the Hedgehog pathway. In addition, they were associated with axon guidance, GAGs biosynthesis, and basal cell carcinoma (Figure 6B). Finally, the analysis also revealed a statistically significant co-citation based on scientific text cited in PubMed, in which we observed some of the publications were associated with the role of the Hedgehog pathway in gastric, bladder, glioma, colon, pancreatic, and mesothelioma tumors as well as CSCs (Table 10).

4 | DISCUSSION

Using publicly available gene expression data, we showed in a large cohort of breast cancer patients that the expression of the Hh-related genes SHH, HHAT, PTCH1, GLI1, GLI2, and GLI3 of the hedgehog pathway was associated with better relapse-free survival of the patients. However, the expression of the GLI transcription factors has a subtype-specific negative impact on the prognosis of the patients, especially in HER2-positive (GLI1-3), PR-negative (GLI1), and grade 3 tumors (GLI3). When we compared the expression of all analyzed genes of the Hedgehog pathway between non-transformed and cancerous breast tissue, we found that they were more highly expressed in cancerous tissue. In accordance with our findings, the immunohistochemical analysis of SHH, PTCH1, and GLI1 in 52 breast cancer tissues, revealed that staining of GLI was high in all analyzed tumors when compared with adjacent normal tissue. In another study, SHH, DHH, IHH, PTCH1, SMO, and GLI1 were highly expressed in 150 breast tumor samples compared with respective normal samples. The authors also showed that the expression of the aforementioned factors was overexpressed in the patients with Luminal B and triple-negative tumors, while the expression of SHH, DHH, and GLI1 correlates with poor survival. In contrast to the results of our analysis, which revealed that a high expression of SHH, HHAT, PTCH1, GLI1, and GLI2, and GLI3 expression was associated with a better relapse-free survival in the patients if not further stratified (n = 3951), as well as if stratified for Luminal A (n = 1933), Luminal B (n = 1149), and basal-like subtype (n = 618), and partially also in lymph node-positive tumors (n = 1133), in other studies, the expression of these genes has been associated with a poor prognosis. However, an important difference between our study and others is the number of analyzed patients and the methodology implemented (e.g., mRNA expression vs. immunohistochemistry [IHC]). For example, in a cohort of 279 patients, the staining of Hh ligand by IHC was analyzed. In this study, 34% of the patients showed a strong expression of Hh ligand in tumor cells that was associated with grade 3, a PR-negative, and basal-like subtype. It also correlated with poor prognosis in terms of metastasis formation. Also, in a mouse model of breast carcinogenesis, the overexpression of Hh ligand promoted tumor growth, which also depended on the stroma-interaction. In another work in which the protein levels of GLI1 were analyzed by IHC in 315 patients, it was shown that the expression of GLI1 in tumor epithelial cells correlated with the lymph node status. The survival analysis showed that high expression of GLI1 was associated with shorter disease-free survival in the patients with ER-negative status and lymph node-positive status, as well as with grade 3, and Her2-positive tumors. Consistently, in our study, we also found that GLI1 expression correlates with worse relapse-free survival in HER2+ tumors. In 108 biopsies of HER2-positive breast cancer treated patients the nuclear staining of GLI1 was associated with poor prognosis. Using a data set that contained the clinical data of 508 breast cancer patients, Rudolph et al reported that the poor outcome of patients with Luminal A tumors correlated with high GLI1 expression. Besides this, the co-expression of GLI1 with EGFR and SNAI1 was associated with poor distant disease-free survival in Luminal A tumors. These data are very important and interesting because patients with the Luminal A subtype have a good prognosis, but some of them (14%) suffer from a disease recurrence within 3–5 years of diagnosis and have a risk of long-term recurrence. Probably, the expression of GLI1 has some impact on the recurrence of these patients. In triple-negative breast tumors that have the worst outcome in patients, a high expression of Hh ligand correlates with a poor overall survival. Also, it has been demonstrated that postranscriptional, post translational as well as splicing variants could affect the signaling of the Hedgehog pathway. For example, to activate the pathway, Smo is released and begins the postranslational mechanism of the GLI factors.
Interestingly, whilst GLI1 acts as a transcriptional activator, GLI3 acts as a repressor, but depending on posttranscriptional and posttranslational modifications, GLI2 can be an activator or repressor. Consequently, the expression of the target genes (PTCH1 and GLI1) depends on this mechanism. Also, it has been described that the posttranslational modifications of GLI1 proteins are associated with the induction and progression of different types of cancer in which GLI is highly expressed. Some studies showed that miRNAs affect the expression of genes of the Hedgehog pathway such as GLI1, GLI2, and GLI3 via posttranscriptional mechanisms, thereby inhibiting malignant processes including proliferation, CSC renewal, and invasiveness of retinoblastoma, medulloblastoma, prostate cancer cells. Also, it has been shown that splice variants of PTCH1 and PTCH2 have different functions in the activation of Hedgehog signaling.

The role of heparan sulfate sulfotransferase enzymes in the initiation and progression of cancer is an emerging topic in the breast cancer research field. Our group and others have demonstrated the importance of HS2ST1 and HS3ST2 in the triple-negative breast cancer cell line MDA-MB-231 correlates with a decrease in the frequency of the CSC population CD44+/CD24-/low and an increase in the ALDH activity, while in the luminal cell line MCF-7 no changes were observed in the stem cell frequency, whereas the ALDH1 activity was reduced. A change in the expression pattern in dependency on the cell line and sulfotransferase type was also observed for various genes associated with the Wnt and Notch pathway, as well as in the expression of some syndecans and heparanase. Also, it has been demonstrated that HS2ST1 and HS3ST2 regulate the Mitogen-activated protein kinase (MAPK) pathway and the invasion, proliferation, and senescence of breast cancer cells. Interestingly, HS2ST1 inhibits cell invasion while HS3ST2 increases invasiveness, transendothelial migration, and motility of triple-negative cells, whereas luminal cells become less invasive. Indeed, it was shown that the HS3ST2 gene is epigenetically silenced particularly in the luminal B subtype of breast cancer compared to healthy breast tissue. These data demonstrate that it is important to study heparan sulfate sulfotransferases to learn more about their role in the progression of breast cancer and other types of cancers. One should take into consideration that their function depended on the type of cell and the enzyme studied.

As previously mentioned, the interaction of SHH and ELP1 has been shown to influence the prognosis of pediatric medulloblastoma. In our study, we have observed that the co-expression of ELP1 and SHH was associated with a poor prognosis in the breast cancer patients with HER2+ tumors, suggesting a possible link between SHH and ELP1 also in this tumor entity. It has been shown that in melanoma ELP3, ELP5, and ELP6 are upregulated inducing the aggressiveness of the tumor cells. In a mouse breast cancer model, the expression of ELP3 was associated with invasion and metastasis formation. These results suggest that the elongation complex may be an important factor in the induction and progression not only of breast cancer but also of other types of cancer.

Our analysis of the expression of the genes related to the Hh pathway showed that all genes were expressed by all of the cell lines, however, the HER2-positive and BCLC subtypes expressed the pathway constituents most strongly. In particular, the GLI transcription factors were more expressed in the basal cell lines. In line with these results, Koike et al. showed that the expression of GLI1 and GLI2 were higher in triple-negative compare with ER-negative cell lines. In the same study, upon the inhibition of the Hh pathway the tumor growth and the frequency of CSCs was diminished. In other studies, a high expression of GLI1 was associated with the expression of EMT and CSC markers, as well as with the invasion capacity in triple-negative breast cancer cell lines. GLI1 increases the invasive properties of triple-negative cells by up-regulating the expression of metalloprotease 1, vascular endothelial growth factor-A (VEGFA), and CD24. The induction of stemness markers by GLI1 is not only restricted to the triple-negative subtype, but was also found in ER-positive and HER2-positive tumors.

In the network analysis, we observed that genes associated with the Hh pathway were associated with different signaling pathways including cAMP, proteoglycans, and other signaling pathways in cancer. The interaction of the Hedgehog pathway with other signaling pathways has been shown to promote progression of breast cancer and other types of cancers. For example, the cAMP pathway has been associated with solid and hematological tumors, having either a negative or positive impact on the patients’ outcome in dependency on the type of cancer. Ramaswamy et al described that inhibiting the PI3K/Akt pathway down-regulates the levels of SMO and GLI1. Consequently, they observed a decrease in tumor growth in a murine xenograft model. The transcription factor forkhead box C1 (FOXC1) which is induced by NF-κB, upregulates the expression of GLI2 in basal-like breast cancer cells increasing the frequency of CSCs. Moreover, in breast tissues, the protein levels of FOXC1 correlated with the protein levels of GLI2 and PTCH1. In other tumors, the expression of GLI1 also depends on the signaling of K-Ras, c-Myc, Wnt-beta.
catenin, and TGFβ.\textsuperscript{77} Akt-GSK3β activation by osteopontin results in the nuclear localization and the activation of GLI1, which in turn upregulates the expression of EMT markers and induces chemoresistance in breast cancer cell lines.\textsuperscript{68} Also, proteoglycans regulate the signaling of the Hh pathway (reviewed in Karamanos et al.\textsuperscript{46}).

In conclusion, our data have shown that the expression of several genes related to the Hedgehog pathway is associated with better survival of breast cancer patients. Interestingly, on the one hand, we found that the GLI (1, 2, and 3) transcription factors emerge as important therapeutic targets by negatively influencing the survival of the patients with HER2-positive tumors. On the other hand, GLI1 correlates with worse relapse-free survival of patients with PR-negative tumors and GLI3 for patients with grade 3 tumors. Previously, it was observed that the inhibition of Hedgehog acetyl transferase, an important activator of the HH pathway, significantly affected the proliferation of HER2-positive breast cancer cells.\textsuperscript{79} On the other hand, in gastric cancer cells the inhibition of HER2 with trastuzumab diminished the expression of GLI1 drastically. In the same study, the authors found that the inhibition of HER2 and SMO has a synergistic effect on the viability of the gastric cancer cells. The authors suggested that the combination of trastuzumab and GLI1 and/or SMO inhibitor could be important for the treatment of patients with HER2-positive gastric cancer.\textsuperscript{79} Interestingly, in esophageal adenocarcinoma, in which the patients also show an amplification of HER2, it was demonstrated that the pharmacological inhibition of HER2 by trastuzumab, and PI3K–mTORC1 by BEZ235 downregulated the expression of GLI1. The inhibition of HER2/PI3K–mTORC1 reduced the capacity of proliferation of esophageal cancer cells. However, this effect was intensified by combination with the GLI1/2 inhibitor GANT61.\textsuperscript{80} Further studies are needed to validate that the pharmacological inhibition of HER2 significantly affects the HH pathway and consequently the aggressiveness of tumor cells, to corroborate that blocking these pathways could improve the survival of patients with HER2-positive tumors. Considering the findings discussed above, we believe that our study suggests that indeed the Hedgehog pathway significantly affects the prognosis of patients with HER2-positive breast cancer. Moreover, we have demonstrated that the heparan sulfate sulfotransferases HS2ST1 and HS3ST2 have a differential impact on breast cancer patient survival and modulate the expression of selected constituents of the Hedgehog pathway in a cell type-specific manner. Finally, our data suggest that – analogously to findings in medulloblastoma – ELP1 appears to be associated with the hedgehog pathway at least in HER2+ breast cancer.

To date, Hedgehog pathway inhibitors have been used in different types of cancer with promising results.\textsuperscript{81} However, we still need to know more about the Hedgehog pathway. Of particular interest appear the mechanisms by which it communicates with other pathways, the impact it has on breast cancer and other cancer types, and the development of specific inhibitors of the Hh pathway. As heparan sulfate modulates Hh signaling,\textsuperscript{8,46} the use of heparinoids or heparan sulfate-related drugs may expand the therapeutic repertoire for targeting the hedgehog pathway in the future.\textsuperscript{45} As a perspective of this study, it is necessary to validate the role of Hedgehog in HER2-positive, PR-negative, and grade 3 tumors in the induction, progression, and treatment of breast cancer. Based on this study, we expect that more tools will be available to predict the progression and to treat this devastating disease in a patient-tailored and subtype-specific manner.

**ACKNOWLEDGMENTS**

We would like to acknowledge Birgit Pers for expert technical assistance. We acknowledge funding by a German Academic Exchange Service (DAAD) Research Grant – Short Term Grant 91749472 (to NAE-S.), Conselho Nacional de Desenvolvimento Científico e Tecnológico (grants 290231/2017-5–SWE to FCOBT and 302171/2018-8-PQ to MSGP), and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) (to MSGP), EU Horizon 2020 project RISE-2014, action No. 645756 “GLYCANC” (to MG and MSGP), DFG Research Grants – GR4743/5-1 (to BG) and GO 1392/8-1 (to MG). The support of the Higher Education Institutional Excellence Programme (2020-4.1.1.-TKP2020) of the Ministry for Innovation and Technology in Hungary, within the framework of the Bionic thematic programme of the Semmelweis University to BG is gratefully acknowledged. Open Access funding enabled and organized by Projekt DEAL.

**CONFLICT OF INTERESTS**

The authors declare that there are no conflict of interests.

**AUTHOR CONTRIBUTIONS**

Julia Kuehn performed the hedgehog pathway Kaplan-Meier and Real Time-PCR data analyses. Felipe C.O.B. Teixeira performed the PCR analysis on HS sulfotransferase-overexpressing cells. Nancy Adriana Espinoza-Sánchez performed Kaplan-Meier analyses of HS sulfotransferases and ELP1, co-expression Kaplan-Meier analyses, and STRING analysis. Balázs Győrffy provided resources and supervised and supported the Kaplan-Meier analysis. Burkhard Greve co-supervised NA Espinoza-Sánchez and provided expertise in gene expression analysis. Ludwig Kiesel provided resources and clinical expertise. Mauro S. G. Pavao co-supervised,
and Felipe C.O.B. Teixeira provided expertise in HS sulfotransferases. Julia Kuehn, Nancy Adriana Espinoza-Sánchez and Martin Götte prepared the figures. Nancy Adriana Espinoza-Sánchez and M Götte wrote the main manuscript and all authors reviewed and commented on the manuscript. Martin Götte conceived, coordinated and supervised the study.

ORCID
Martin Götte https://orcid.org/0000-0003-2360-2496

REFERENCES

1. Perrimon N, Pitsouli C, Shilo BZ. Signaling mechanisms controlling cell fate and embryonic patterning. Cold Spring Harb Perspect Biol. 2012;4:a005975. https://doi.org/10.1101/cshperspect.a005975
2. Skoda AM, Simovic D, Karin V, Kardum V, Vranic S, Serman L. The role of the Hedgehog signaling pathway in cancer: a comprehensive review. Bosn J Basic Med Sci. 2011;27:513-537.
3. Litingtung Y, Dahn RD, Li Y, Fallon JF, Chiang C. Shh and Gli3 are dispensable for limb skeleton formation but regulate digit number and identity. Nature. 2002;418:979-983.
4. Manikowski D, Jakobs P, Jboor H, Grobe K. Soluble heparin and heparan sulfate glycosaminoglycans interfere with sonic Hedgehog solubilization and receptor binding. Molecules. 2019;24:1607.
5. Vitale D, Kumar Katakan S, Greve B, et al. Proteoglycans and glycosaminoglycans as regulators of cancer stem cell function and therapeutic resistance. FEBS J. 2019;286:2870-2882.
6. Rubin LL, de Sauvage FJ. Targeting the Hedgehog pathway in cancer: beyond Smoothened. Oncotarget. 2015;6:13899-13913.
7. Hui CC, Angers S. Gli proteins in development and disease. Annu Rev Cell Dev Biol. 2001;6:53-79.
8. Taylor MD, Liu L, Raffel C, et al. Mutations in SUFU predispose to medulloblastoma. Nature. 2002;31:306-310.
9. Xie J, Murone M, Luoh SM, et al. Activating smoothened mutations in sporadic basal-cell carcinoma. Nature. 1998;391:90-92.
10. Karhadkar SS, Bova GS, Abdallah N, et al. Hedgehog signalling in prostate regeneration, neoplasia and metastasis. Nature. 2004;431:707-712.
11. Berman DM, Karhadkar SS, Maitra A, et al. Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. Nature. 2003;425:846-851.
12. Sicklick JK, Li YX, Jayaraman A, et al. Dysregulation of the Hedgehog pathway in human hepatocarcinogenesis. Carcinogenesis. 2006;27:748-757.
13. Thayer SP, di Magliano MP, Heiser PW, et al. Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. Nature. 2003;425:851-856.
14. Watkins DN, Berman DM, Burkholder SG, Wang B, Beachy PA, Baylin SB. Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. Nature. 2003;422:313-317.
15. Noman AS, Uddin M, Chowdhury AA, et al. Serum sonic hedgehog (SHH) and interleukin-(IL-6) as dual prognostic biomarkers in progressive metastatic breast cancer. Sci Rep. 2017;7:017-01268.
16. Noman AS, Uddin M, Rahman MZ, et al. Overexpression of sonic hedgehog in the triple negative breast cancer: clinicopathological characteristics of high burden breast cancer patients from Bangladesh. Sci Rep. 2016;6:18830.
17. Espinoza NA, Györfy B, Fuentes-Pananá EM, Götte M. Differential impact of classical and non-canonical NF-κB pathway-related gene expression on the survival of breast cancer patients. J Cancer. 2019;10:5191-5211.
18. Hassan N, Rutsch N, Györfy B, Espinoza-Sánchez NA, Götte M. SETD3 acts as a prognostic marker in breast cancer patients and modulates the viability and invasion of breast cancer cells. Sci Rep. 2020;10:59057.
19. Lewis MT, Ross S, Strickland PA, et al. The Glil2 transcription factor is required for normal mouse mammary gland development. Dev Biol. 2001;238:133-144.
20. Lewis MT, Ross S, Strickland PA, et al. Defects in mouse mammary gland development caused by conditional haploinsufficiency of Patched-1. Development. 1999;126:5181-5193.
21. Lewis MT. Hedgehog signaling in mouse mammary gland development and neoplasia. J Mammary Gland Biol Neoplasia. 2001;6:53-66.
22. Kasper M, Jacobs V, Fisch M, Thoigard R. Hedgehog signalling in breast cancer. Carcinogenesis. 2009;30:903-911. https://doi.org/10.1038/gncarcin.1009.856.
23. Kubo M, Nakamura M, Tasaki A, et al. Hedgehog signalling pathway is a new therapeutic target for patients with breast cancer. Cancer Res. 2004;64:6071-6074. https://doi.org/10.1158/0008-5472.can-04-0416
24. Györfy B, Lanczyk A, Eklund AC, et al. An online survival analysis tool to rapidly assess the effect of 22,277 genes on cancer outcome. Int J Gynaecol Obstet. 2015;131(suppl 1):S36-S39. https://doi.org/10.1016/j.ijigo.2015.03.015
25. Tjan-Heijnen V, Viale G. The lymph node and the metastasis. N Engl J Med. 2018;378:2045-2046. https://doi.org/10.1056/NEJMctbr1803854
26. Thayer SP, di Magliano MP, Heiser PW, et al. Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. Nature. 2003;425:851-856.
34. Szklarczyk D, Gable AL, Lyon D, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* 2019;47:D607-D613.

35. Sørlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A.* 2001;98:10869-10874.

36. Badve S, Dabbs DJ, Schnitt SJ, et al. Basal-like and triple-negative breast cancers: a critical review with an emphasis on the implications for pathologists and oncologists. *Mod Pathol.* 2011;24:157-167.

37. Yamamoto M, Ito T, Shimizu T, et al. Epigenetic alteration of the NF-xB-inducing kinase (NIK) gene is involved in enhanced NIK expression in basal-like breast cancer. *Cancer Sci.* 2010;101:2391-2397.

38. Meric-Bernstam F, Hung MC. Advances in targeting human epidermal growth factor receptor-2 signaling for cancer therapy. *Clin Cancer Res.* 2006;12:6326-6330.

39. Szklarczyk D, Gable AL, Lyon D, et al. STRING v11: protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* 2019;47:D607-D613.

40. Gonzalez-Angulo AM, Morales-Vasquez F, Hortobagyi GN. Overview of resistance to systemic therapy in patients with breast cancer. *Adv Exp Med Biol.* 2007;608:1-22.

41. Li JP, Kusche-Gullberg M. Heparan sulfate: biosynthesis, structure, and function. *Int Rev Cell Mol Biol.* 2016;325:215-273.

42. Teixeira FCOB, Vijaya Kumar A, Kumar Katakam S, et al. The Heparan sulfate sulfotransferases HS2ST1 and HS3ST2 are novel regulators of breast cancer stem-cell properties. *Front Cell Dev Biol.* 2020;8. https://doi.org/10.3389/fcel.2020.559554

43. Hawer H, Hamrie Meister A, Ravichandran KE, Glatt S, Schaffrath R, Klassen R. Roles of elongator dependent tRNA modification pathways in neurodegeneration and cancer. *Genes.* 2018;10:10.

44. Smith SE, Mellor P, Ward AK, et al. Molecular characterization of breast cancer cell lines through multiple omic approaches. *Breast Cancer Res Treat.* 2017;19:017-0855.

45. Espinoza-Sánchez NA, Götté M. Role of cell surface proteoglycans in cancer immunotherapy. *Semin Cancer Biol.* 2020;62:48-67.

46. Karamanos NK, Piperigkou Z, Theocharis AD, et al. Proteoglycan chemical diversity drives multifunctional cell regulation and therapeutics. *Chem Rev.* 2018;118:9152-9232.

47. Riaz SK, Khan JS, Shah STA, et al. Involvement of hedgehog pathway in early onset, aggressive molecular subtypes and metastatic potential of breast cancer. *Cell Commun Signaling.* 2018;16:3. https://doi.org/10.1186/s12974-017-0213-y

48. Liu S, Duan X, Xu L, et al. Nuclear Gli1 expression is associated with pathological complete response and event-free survival in HER2-positive breast cancer treated with trastuzumab-based neoadjuvant therapy. *Tumour Biol.* 2016;37:4873-4881.

49. O’Toole SA, Machalek DA, Shearer RF, et al. Hedgehog overexpression Is associated with stromal interactions and predicts for poor outcome in breast cancer. *Cancer Res.* 2011;71:4002-4014. https://doi.org/10.1158/0008-5472.can-10-3738

50. Ramaswamy B, Lu Y, Teng K-Y, et al. Hedgehog signaling is a novel therapeutic target in tamoxifen-resistant breast cancer aberrantly activated by PI3K/AKT pathway. *Cancer Res.* 2012;72:5048-5059. https://doi.org/10.1158/0008-5472.can-12-1248

51. Ribelles N, Perez-Villa L, Jerez JM, et al. Pattern of recurrence of early breast cancer is different according to intrinsic subtype and proliferation index. *Breast Cancer Res.* 2013;15:R98. https://doi.org/10.1186/bcr3559

52. Rudolph M, Sizemore ST, Lu Y, et al. A hedgehog pathway-dependent gene signature is associated with poor clinical outcomes in Luminal A breast cancer. *Breast Cancer Res Treat.* 2018;169:457-467. https://doi.org/10.1007/s10549-018-4718-x

53. Sasaki H, Nishizaki Y, Hui C, Nakafuku M, Kondoh H. Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. *Development.* 1999;126:3915-3924.

54. Pietrobono S, Gagliardi S, Stecca B. Non-canonical Hedgehog signaling pathway in cancer: activation of GLI transcription factors beyond smoothened. *Front Genet.* 2019;10:556.

55. Miele E, Po A, Begalli F, et al. b-arrestin1-mediated acetylation of Gli1 regulates Hedgehog/GLi signaling and modulates self-renewal of SHH medulloblastoma cancer stem cells. *BMC Cancer.* 2017;17:488.

56. Chen S, Zhang G, Yu Q, Zhang X, Han G. Mir-361 inhibited prostate carcinoma cell invasion by targeting GLI1. *Int J Clin Exp Pathol.* 2017;10:6108-6116.

57. Zhao D, Cui Z. MicroRNA-361-3p regulates retinoblastoma cell proliferation and stemness by targeting hedgehog signaling. *Exp Ther Med.* 2019;17;1154-1162.

58. Rahnama F, Toftgård R, Zaphiropoulos PG. Distinct roles of PTCH2 splice variants in Hedgehog signalling. *Biochem J.* 2004;378:325-334.

59. Shimokawa T, Svärd J, Heby-Henricsson K, Teglund S, Toftgård R, Zaphiropoulos PG. Distinct roles of first exon variants of the tumor-suppressor Patched1 in Hedgehog signaling. *Oncogene.* 2007;26:4889-4896.

60. Kang D, Jung SH, Lee GH, et al. Sulfated syndecan 1 is critical to preventing cellular senescence by modulating fibroblast growth factor receptor endocytosis. *EASEB J.* 2020;12:2022-6116.

61. Kassim SK, Shehata HH, Abou-Alhussein MM, Sallam MM, Amin II. Laboratory validation of formal concept analysis of the methylation status of microarray-detected genes in primary breast cancer. *Tumour Biol.* 2017;39:1010428317698390.

62. Rapino F, Delaunay S, Rambow F, et al. Codon-specific translation reprogramming promotes resistance to targeted therapy. *Nature.* 2018;558:605-609.

63. Close P, Gillard M, Ladang A, et al. DERP6 (ELP5) and Delaunay S, Rapino F, Tharun L, et al. Elp3 links tRNA modification to IRES-dependent translation of LEF1 to sustain anti-cancer activities of the non-canonical hedgehog inhibitor GANT61 in triple-negative breast cancer cell lines. *J Biol Chem.* 2012;287:32535-32545.

64. Delaunay S, Rapino F, Tharun L, et al. Elp3 links tRNA modification to IRES-dependent translation of LEF1 to sustain metastasis in breast cancer. *J Exp Med.* 2016;213:2503-2523.

65. Kojima K, Ohta Y, Saitoh W, et al. Anti-cancer stem cell activities of the non-canonical hedgehog inhibitor GANT61 in triple-negative breast cancer cells. *Breast Cancer.* 2017;24:683-693.

66. Lei J, Fan L, Wei G, et al. Gli-1 is crucial for hypoxia-induced epithelial-mesenchymal transition and invasion of breast cancer. *Tumour Biol.* 2015;36:3119-3126.
Colavito SA, Zou MR, Yan Q, Nguyen DX, Stern DF. Significance of glioma-associated oncogene homolog 1 (GLI1) expression in claudin-low breast cancer and crosstalk with the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway. *Breast Cancer Res*. 2014;16:014-0444.

Das S, Samant RS, Shevde LA. Nonclassical activation of Hedgehog signaling enhances multidrug resistance and makes cancer cells refractory to Smoothened-targeting Hedgehog inhibition. *J Biol Chem*. 2013;288:11824-11833.

Kwon YJ, Hurst DR, Steg AD, et al. Gli1 enhances migration and invasion via up-regulation of MMP-11 and promotes metastasis in ERα negative breast cancer cell lines. *Clin Exp Metastasis*. 2011;28:437-449.

Cao X, Geradts J, Dewhirst MW, Lo HW. Upregulation of VEGF-A and CD24 gene expression by the tGLI1 transcription factor contributes to the aggressive behavior of breast cancer cells. *Oncogene*. 2012;31:104-115.

O’Brien CS, Farnie G, Howell SJ, Clarke RB. Breast cancer stem cells and their role in resistance to endocrine therapy. *Horm Cancer*. 2011;2:91-103.

Sun Y, Wang Y, Fan C, et al. Estrogen promotes stemness and invasiveness of ER-positive breast cancer cells through Gli1 activation. *Mol Cancer*. 2014;13:1476-4598.

Wang X, Wei S, Zhao Y, et al. Anti-proliferation of breast cancer cells with itraconazole: Hedgehog pathway inhibition induces apoptosis and autophagic cell death. *Cancer Lett*. 2017;385:128-136. https://doi.org/10.1016/j.canlet.2016.10.034

Fajardo AM, Piazza GA, Tinsley HN. The role of cyclic nucleotide signaling pathways in cancer: targets for prevention and treatment. *Cancers*. 2014;6:436-458. https://doi.org/10.3390/cancers6010436

Chung S, Jin Y, Han B, et al. Identification of EGF-NF-κB-FOXC1 signaling axis in basal-like breast cancer. *Cell Commun Signal*. 2017;15:017-0180.

Han B, Qu Y, Jin Y, et al. FOXC1 activates smoothened-independent Hedgehog signaling in basal-like breast cancer. *Cell Rep*. 2015;13:1046-1058.

Palle K, Mani C, Tripathi K, Athar M. Aberrant GLI1 Activation in DNA damage response, carcinogenesis and chemoresistance. *Cancers*. 2015;7:2330-2351.

Matevossian A, Resh MD. Hedgehog acyltransferase as a target in estrogen receptor positive, HER2 amplified, and tamoxifen resistant breast cancer cells. *Mol Cancer*. 2015;14:72. https://doi.org/10.1186/s12943-015-0345-x

Shao X, Kuai X, Pang Z, et al. Correlation of Gli1 and HER2 expression in gastric cancer: identification of novel target. *Sci Rep*. 2018;10(8):397.

Kebenko M, Drenckhan A, Gros SJ, et al. ErbB2 signaling activates the Hedgehog pathway via PI3K-Akt in human esophageal adenocarcinoma: identification of novel targets for concerted therapy concepts. *Cell Signal*. 2015;27:373-381.

Peer E, Tesanovic S, Aberger F. Next-generation Hedgehog/GLI pathway inhibitors for cancer therapy. *Cancers*. 2019;11:538. https://doi.org/10.3390/cancers11040538

---

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

---

**How to cite this article:** Kuehn J, Espinoza-Sanchez NA, Teixeira FCOB, et al. Prognostic significance of hedgehog signaling network-related gene expression in breast cancer patients. *J Cell Biochem*. 2021;122:577–597. https://doi.org/10.1002/jcb.29886