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Evaluating the correlation of Hedgehog pathway with Claudin-1 expression in invasive breast cancer

Daping Chen, Xing Cheng*

Department of thyroid and breast surgery, Xianning Central Hospital, The First Affiliated Hospital of Hubei University of Science and Technology, Xianning, Hubei437000, China.

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ABSTRACT

Being a heterogeneous disease, breast cancer illustrates different biological and phenotypic features which make its diagnosis and treatment challenging. This study aimed to evaluate the expression levels of crucial components of the Hedgehog signaling pathway, the correlation between the signal transducer Smo, and clinicopathologic features (lymph node metastasis and metastasis stage) in invasive breast carcinoma. Besides, the inverse correlation was considered between expression levels of Smo and Claudin-1. For this purpose, in a case-control study, we evaluated 72 tumor and adjacent normal tissue specimens obtained from invasive ductal breast cancer patients. The expression levels of Hedgehog signaling components (Smo, Gli1, and Ptch), Claudin-1, E-cadherin, and MMP2 were measured by qRT-PCR. The correlations between Smo expressions with some clinicopathologic parameters were also analyzed. Compared to normal adjacent tissues, the results showed up-regulation of Hedgehog signaling in invasive breast carcinoma samples. Upregulation of the signal transducer Smo correlated with tumor stages and lymph node metastasis of the breast tumors. This correlation was affected by the expression of Her2. A significant correlation existed between expression levels of the signal transducer Smo and Claudin-1, E-cadherin as an epithelial cell marker, and MMP2 as a metastasis-related gene in advanced metastatic tumor samples. Based on the obtained results, a new layer of molecular complexity was found, which should be considered in managing patients with invasive breast carcinoma. The results suggested a key role for Hedgehog signaling in invasive breast carcinoma. In terms of the inverse correlation between expression levels of Claudin-1 and Hedgehog signaling, Claudin-1 could serve as a candidate gene in diagnostic studies. Thus, its clinical significance should be further clarified.

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Introduction

Breast cancer is the most common cancer among women all over the world (1). In managing, diagnosing, and treating this malignancy, various clinical and pathological factors have prognostic significance in patient survival (2). These factors include the patient's age, tumor size, menopause status, involvement of lymphatic vessels, hormone receptor status, overexpression of the human epidermal growth factor receptor (Human epidermal growth factor 12: Her), and histological features such as the stage and grade of the tumor (3, 4). Although the determination of these predictive algorithms in diagnosing cancer risk has improved the survival rate of patients, one of the crucial challenges in this field is that this approach does not consider the molecular complexities of each neoplasm (5). It seems that accurate prediction of tumor metastasis potential is necessary for breast cancer patients' diagnostic and therapeutic management (1, 6).

The hedgehog signaling pathway is an evolutionarily conserved pathway that plays an essential role in vertebrate embryogenesis by controlling cell fate, patterning, proliferation, survival, and differentiation. In adults, this pathway regulates tissue homeostasis, regeneration, and maintenance of stem cells. The hedgehog signaling pathway is activated by the binding of protein ligands [Shh (Sonic Hedgehog), Ihh (Indian Hedgehog), and Dhh (Desert Hedgehog)] to the Ptch membrane receptor. The Ptch receptor is a negative regulator of another membrane receptor called Smo (7). After ligand binding, the inhibitory effect of Ptch on Smo is removed. This event causes the transformation of Smo and subsequent induction of transcription factors Gli1, Gli, Cli, and Gli3 (8, 9). The activity of these transcription factors causes the transcription of hedgehog-responsive genes such as Gli, cyclin 1, and B2. Up-regulation of the Hedgehog signaling pathway has been reported in tumorigenesis and metastasis of various types of cancer (10). The importance of this signaling pathway in carcinogenesis becomes more evident when it is noted that many genes involved in this signaling pathway, such as Gli1, Shh, Smo, and Gli2, act as carcinogens, and Ptch receptor acts as a tumor suppressor (11).

Epithelial to mesenchymal transition (EMT) is a phenotypic change related to the process of metastasis and is considered a risk factor for breast cancer. During EMT, an expression change from E-cadherin (epithelial marker) to N-cadherin (mesenchymal marker) is seen, and this event causes a change in extracellular matrix adhesion (12).

* Corresponding author. Email: chengxing1628@163.com

Acquisition of invasive phenotype has many similarities with EMT, including the loss of cell-cell junctions and increased cell motility. It has been reported that tumors with decreased E-cadherin expression have more metastasis and invasion to lymph nodes than tumors without changes in the expression levels of E-cadherin (13).

There is a well-founded connection between the role of proteins involved in tight junctions and cancer development. Changes in the structure and function of tight junctions have been reported in the development of carcinoma in different organs. In this regard, it has been determined that the absence or defect of these connections is directly related to the development of neoplastic phenotype in epithelial cells (1). The Claudin family is made of membrane proteins involved in tight junctions (14). Among these, Claudin 1 is a gene that has a high expression during the early development of mammary glands. This expression is highly regulated during different stages of the development of normal mammary glands—studies conducted on aggressive breast cancer and some cell lines. Breast cancer refers to the reduction or complete absence of claudin-1 gene expression. Also, a correlation between this decrease in expression and recurrence has been reported (15).

Since the Hedgehog signaling pathway is overregulated in breast carcinoma (16), the present study examines the relationship between the overregulation of this pathway with different stages of the disease and metastasis to lymphatic vessels. Also, because the Claudin-1 gene is one of the critical members of tight cell junctions, the expression levels of this gene and the signal transmitter Smo signal transducer Smo in the Hedgehog pathway in Invasive ductal breast carcinoma with different activity statuses of 2-Her are investigated. The inverse expression relationship between the Hedgehog pathway and Claudin 1 gene is possible. It is essential to predict metastasis to lymphatic vessels in breast cancer.

Materials and Methods

Patients

In the present case-control study, 36 pairs of tumor and adjacent healthy tissue samples from patients with invasive ductal carcinoma of the breast were obtained from the tumor bank of the hospital's cancer institute. The sampling of patients was accompanied by informed consent before surgery. The report of the clinical-pathological features of the patients, including grading and staging (including tumor size, lymphatic vessel status, hormone receptor status, and Her-2 expression status) for tissue samples, are summarized in Table 1. Tissue samples were stored in liquid nitrogen until molecular analysis.

Tissue RNA extraction and cDNA synthesis

To check the expression of the studied genes, total RNA was extracted from the tissue using a Trizol reagent (Invitrogen, USA). To remove possible contamination with genomic DNA, the extracted RNA was treated with a DNase enzyme. The quality of extracted RNA was confirmed by electrophoresis on agarose gel, and its concentration and purity were measured by spectrophotometry with optical absorption at 260 and 280 nm wavelengths. DNA synthesis was performed by reverse transcription of 3 µg of total RNA extracted by random hexamer and reverse transcription enzyme Takara (PrimeScript TM Tase (Japan).

Quantitative real-time PCR reaction

Oligonucleotide primers for Gli1, Smo, MMP2, E-cadherin, Claudin-1, Ptch, and GAPDH genes were designed according to Table 2. The specificity and uniqueness of the primer sequence in the human genome were evaluated using Blast software (http://blast.ncbi.nlm.gov/blast.cgi). The reaction mixture was prepared in a final volume of 20 microliters, including 10 microliters of Takara (SYBR Green I Master Mix, Japan), 5 picomoles of each primer, 7 microliters of water, and 5 nanograms of synthesized DNA.

The Q-RT-PCR reaction for each gene was performed in two series simultaneously, and each gene's average Ch (threshold cycle) was calculated. The reaction was performed in the ABI StepOne Sequence Detection System (Applied Biosystems, USA) under the following temperature and time conditions:

At first, 95°C for 5 minutes was considered the initial denaturation stage. Then the following temperature program was repeated in 40 cycles.

Denaturation step at 95°C for 10 seconds and annealing/extension steps at 60°C for 30 seconds. Each amplification step was followed by a dissociation step consisting of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s to analyze the melting curve.

The amounts of transcripts of the target genes compared to the expression of the GAPDH gene were calculated as Housekeeping and using the $2^{-\Delta\Delta C}$ formula. The amplification efficiency of the studied genes is based on the slope of the standard curve, which is drawn based on the logarithmic values of successive dilutions of cDNA against the corresponding Cts, according to the formula Efficiency ($E= 10^{-1/slope}$) was calculated.

Statistical analysis of data

Data were presented as mean and standard deviation from at least two independent experiments, and a t-test was used for statistical analysis of data changes. P-values, smaller than 0.05, were considered statistically significant.

Results

Amplification efficiency and melting curve analysis

To determine the efficiency of the amplification reaction for each of the genes, the standard curve that was prepared using serial dilutions of synthesized cDNA samples was used, and all the amplification efficiencies of the studied transcripts were almost equal. It indicates the validity of the quantitative test for the relative evaluation of gene expression. Since the dye SYBR Green I, which is used to identify the PCR product, is attached to any double-stranded DNA and cannot distinguish a specific product from a non-specific one, the presence of things such as primer dimer or non-specific effect also causes an increase in light intensity. It becomes fluorescent. Therefore, to verify the correctness of the reproduced fragment and ensure the absence of non-specific products, the melting curve was used. In this diagram, the specificity of the PCR product was determined due to the presence of the only peak observed for each gene at its unique melting temperature (Figure 1).

Table1. Clinical and pathological features of breast cancer patients.

Characteristics	Number	Percent
Age of onset		
Average (range): 48.65 (29-77)	36	
50 years>	20	56
50 years≤	16	44
Age at onset of menstruation (Mean ± SD)	13.44 ± 1.2	
Age at first delivery (Mean ± SD)	20.7 ± 3.7	
Menopause Status		
Before menopause	23	63
After menopause	13	36
Age at menopause (Mean ± SD)	50.6 ± 3.5	
Previous use of hormones		
Yes	19	53
No	17	47
Tumor size (cm)		
3>	16	44
3≤	20	56
Primary tumor (T stage)		
T1:≤ 2cm	11	31
T2: > 2cm ≤ 5cm	16	44
T3: > 5cm	9	25
Regional lymph nodes (stage N)		
NX	3	8
N0	10	28
N1	7	19
N3	11	31
N4	5	14
Distant metastasis (stage M)		
MX	3	8
M0	14	39
M1	19	53
Metastasis to lymphatic vessels		
Positive	22	61
Negative	14	39
Tumor Stage		
I + II	19	53
III + IV	17	47
Estrogen receptor status		
Negative	11	31
Positive	22	61
Unknown	3	8
Progesterone receptor status		
Negative	12	34
Positive	21	58
Unknown	3	8
Her2 status		
Negative	16	44
Positive	18	50
Unknown	2	6

Table2. Oligonucleotide primers used in Real-Time PCR assay.

Gene		Primer Sequences	Length (base pair)
Ptch	Forward	5'-CGGCAGCCGCGATAAG-3'	75
	Reverse	5'- TTAATGATGCCATCTGCATCCA-3'	
Smo	Forward	5'- GTGCTGGCCCCAATCG-3'	142
	Reverse	5'- GCAGCATGGTCTCGTTGATCT-3'	
Gli1	Forward	5'- GTTCACATGCGCAGACACACT-3'	81
	Reverse	5'- TTCGAGGCGTGAGTATGACTTC-3'	
Claudin-1	Forward	5'- TTGGGCTTCATTCTCGCCTT-3'	214
	Reverse	5'- TTGCTTGCAATGTGCTGCTC-3'	
MMP2	Forward	5'- GGATGCCGCCTTTAACTGGA-3'	203
	Reverse	5'- AGGCACCCTTGAAGAAGTAGC-3'	
E-cadherin	Forward	5'- TCATGAGTGTCCCCCGGTAT-3'	240
	Reverse	5'- TCTTGAAGCGATTGCCCCAT-3'	
GAPDH	Forward	5'- TCACCCACTCCTCCACCTTTG-3'	112
	Reverse	5'- TCCACCACCCTGTTGCTGTAG-3'	

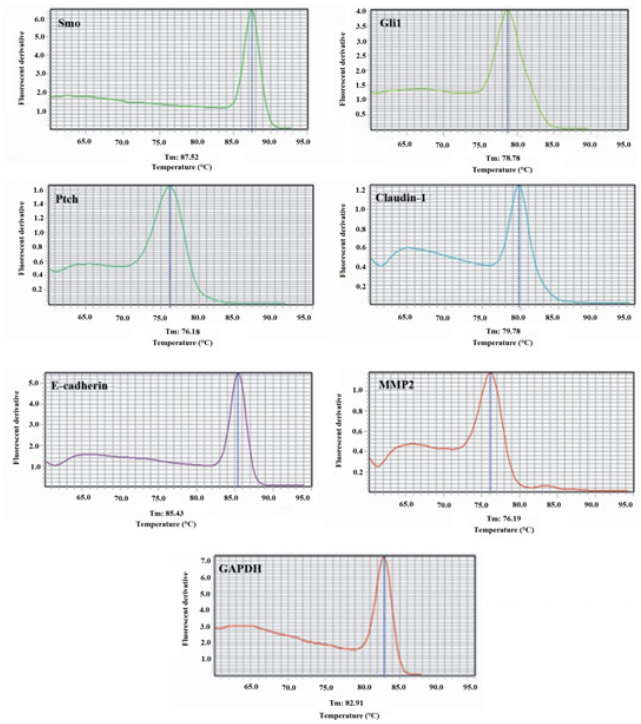

Figure 1. Melting curves of studied genes; the presence of only one peak for each gene at its specific melting temperature indicates the specificity of the amplification product.

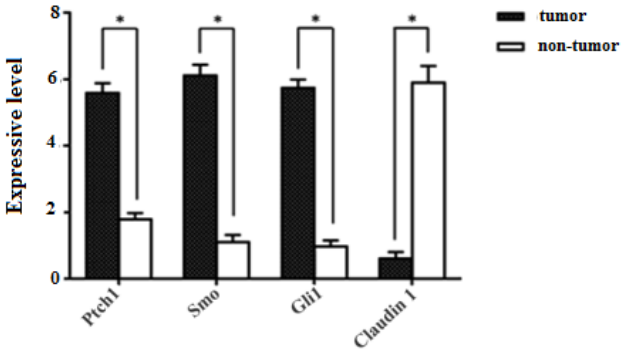

Figure 2. High expressions of the critical members of the Hedgehog oncogenic pathway in tumor and non-tumor invasive breast cancer samples. The transcript expression levels of Gli1, Smo, and Ptch genes are significantly higher in tumor samples. Also, the expression of 1-claudin in tumor samples is lower than the adjacent non-tumor findings.

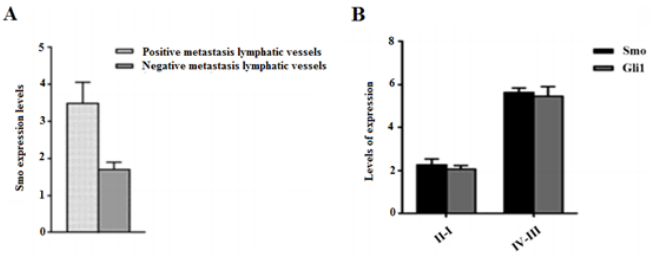

Figure 3. Noticeable difference in transcript levels of key members of the Hedgehog messenger pathway in invasive ductal carcinoma of the breast with different clinical and pathological characteristics. (A): metastasis to lymphatic vessels; (B): Stage of tumor metastasis.

Expression of critical members of the Hedgehog pathway and Claudin-1 gene in breast cancer samples and adjacent healthy tissues

QRT-PCR was used to investigate the expression level of the key members of the Gilmo Hedgehog and Ptch signaling pathway and to investigate the expression levels of the cell junction gene Claudin 1. For this purpose, 36 tumor and non-tumor tissue samples from cancer patients' breasts were examined. As shown in Figure 2, the expression levels of Gli1, Smo, and Ptch transcripts were significantly higher in tumor samples than in adjacent non-tumor samples. The expression level of Claudin1 in tumor samples was significantly lower than in adjacent non-tumor tissues.

Hedgehog signaling pathway activity in breast cancer tumor tissues with different clinic-pathological parameters

We investigated the relationship between the activity levels of the hedgehog signaling pathway and clinical-pathological parameters, the expression levels of two key members of this pathway (Smo and Gli1) among different groups. Figure 3A shows Smo gene expression levels are higher in tumor samples with metastasis to lymphatic vessels. In this regard, there is a significant difference

between stages II and VIII of breast carcinoma tissue (Figure 3B). Also, to determine that the association between high expression of the Smo gene and metastasis to lymphatic vessels is related to the positive or negative status of estrogen and progesterone receptors. The expression of this gene was investigated in different patients, and it was found that the association of the Smo gene and metastasis to lymphatic vessels is not related to the expression status of hormone receptors.

Examining the expression of Smo and Claudin-1 genes in Her-2 positive breast cancer

Previous research has shown that the positive regulation of the Hedgehog signaling pathway is necessary for the proper development of mammary glands and to prevent tumor formation (17). Since Her-2 is a well-known marker related to the metastatic potential of breast cancer, the present researchers investigated whether the relationship between Smo and metastasis to lymphatic vessels is affected by the positive or negative expression of Her-2 compared to breast cancers. With the expression of Her-2 negative in all breast tumor tissues where Her-2 was positive, the expression of Smo increased significantly (Figure 4). These results show a significant positive relationship between the expression of the Her-2 gene and the Smo gene as a critical member and messenger in the Hedgehog pathway.

Also, the expression levels of the Claudin-1 gene, one of the essential components of tight junctions in epithelial cells, was slightly increased in Her-2 positive breast carcinoma samples compared to tumor tissues that lacked Her-2 gene expression.

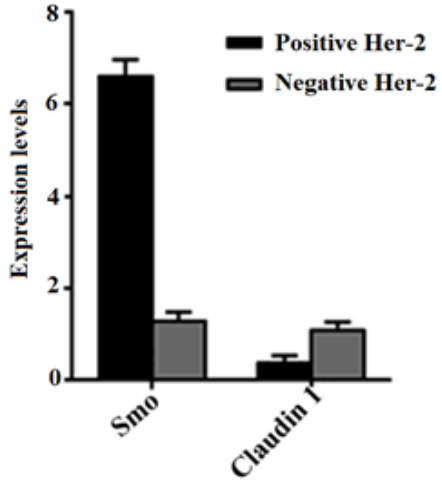

Figure 4. Examining the expression of Smo and Claudin-1 genes in Her-2 positive breast cancer, Smo gene expression levels are significantly higher in Her-2 positive samples than in Her-2 negative samples. Also, the expression levels of the Claudin-1 gene in the Her positive samples have a slight increase compared to Her-2 negative samples.

Investigating the relationship between the expression levels of Smo and 1-Claudin genes and some genes related to metastasis (E-cadherin, MMP2)

Since reports are showing that the overregulation of the Hedgehog pathway can alter the ability of cancer cells to adhere to the epithelium and invade through the extracellular matrix components, examining the expression levels of E-cadherin genes (as a marker of epithelial cell expression) it decreases in the process of metastasis) and MMP2 (a key metalloproteinase whose expression increases in the process of metastasis) was considered in breast cancer samples. In this regard, increased expression of Smo and MMP2 genes was observed in breast tumor samples in the advanced stages of stage IV III metastasis compared to the early stages of stage III. As expected, the expression levels of the Claudin 1 gene and E-cadherin gene in the advanced stages of metastasis decreased compared to earlier stages (Figure 5).

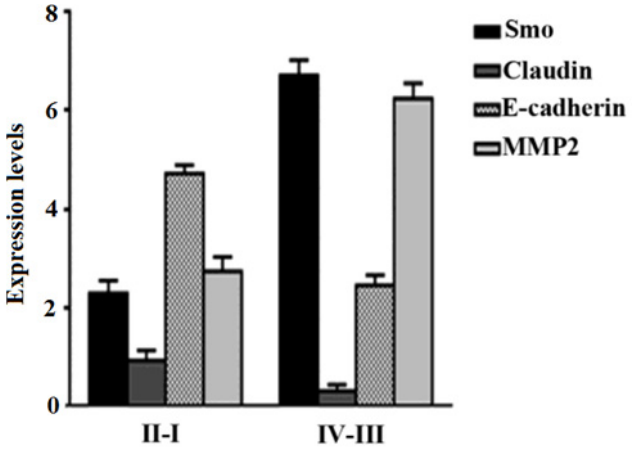

Figure 5. Expression of Smo and 1-Claudin genes and metastasis-related genes (MMP2 and E-cadherin) in breast carcinoma tissues in different stages of metastasis, expression of Smo and MMP2 genes in advanced stages of metastasis (stages IV and III) compared to early stages (stages II and I)) has a more significant increase. On the contrary, the expression levels of 1-Claudin and E-cadherin genes are decreased in the advanced stages of metastasis compared to the early stages.

Discussion

Breast cancer is one of the most common causes of death due to cancer in women around the world, which is a heterogeneous disease with different biological and phenotypic characteristics, and these characteristics have made the diagnosis of this disease challenging. About 90% of people living with breast cancer die due to metastasis and invasion, so identifying the molecular pathways involved in this process can provide new opportunities in diagnosing and treating this malignancy (18-20). The role of the Hedgehog signaling pathway as an essential factor has been proven in tumor growth and development in various cancers, especially breast carcinoma. So, the overexpression of this signaling pathway is considered a prerequisite for the proliferation of breast cancer cells. In this regard, Kasper et al. (3) found that inhibition of the Hedgehog signaling pathway in breast tumors may cause two results. The first is that changing the tumor stroma interferes with the progress of cancer, and the second is that it reduces the power of maintaining cancer stem cells in the tumor tissue.

Due to the key role of the Hedgehog pathway in regulating breast cancer metastasis, this study examined the expression levels of key members of this signaling pathway in patients with invasive ductal carcinoma of the breast, as well as the expression of genes involved in the process of metastasis, including Claudin-1 (an important gene in-

volved in tight intercellular junctions), E-cadherin (as an indicator of epithelial cells) and MMP2 (a key metalloproteinase in the process of metastasis), were investigated. In addition, the present study investigated the relationship between Smo upregulation and decreased expression of the Claudin-1 gene with increased metastasis potential in breast carcinoma.

This study showed that the expression of genes involved in the Hedgehog signaling pathway, including Smo, Ptch, and Gli1, increased significantly in breast cancer tissues, and the expression of the Claudin-1 gene showed a significant decrease in these tissues. This finding was consistent with previous studies. Lu et al. (16), by comparing the expression levels of Claudins in different types of breast cancer, found that low expression levels of Claudin are strongly related to the phenomenon of metastasis and recurrence of the disease and mentioned the expression of this gene as a predictor factor for the repetition of the disease. Also, Kubo et al., by reporting the increased expression levels of the Hedgehog pathway in the tissues of breast cancer patients, referred to this pathway as a new target in treating breast cancer patients (21). Therefore, considering the involvement of the Hedgehog signaling pathway and 1-claudin in the process of metastasis, these results can indicate a significant relationship between the overregulation of the Hedgehog signaling pathway and the reduction of 1-claudin expression in invasive breast cancer. Also, the current researchers found that higher expression levels of Smo were seen in patients with metastasis to lymphatic vessels and were in more advanced stages of metastasis. These data are consistent with previous reports that have suggested that Hedgehog pathway overregulation is associated with survival in breast carcinoma patients.

Overexpression of the Her-2 gene has been detected in more than 30% of breast cancers, which has a relatively poor prognosis (6). Since Her-2 is a known biomarker with the potential to increase metastasis in breast cancer (22), this study investigated whether the relationship between Smo gene expression and metastasis to lymphatic vessels is affected by positive or negative expression of Her-2. Her-2 had higher levels of Smo transcripts compared to Her-negative tissues. These results suggest that 2-Her signaling in breast cancer may be the reason for the significant upregulation of the Smo gene.

Decreased expression of E-cadherin is often reported in invasive cancers, and its absence is significantly associated with the epithelial-to-mesenchymal transition (EMT) process (23, 24). Acquisition of invasive phenotype has similarities with EMT, including loss of cell-cell adhesion and increased cell mobility. During EMT, the switch in the expression of the cadherin gene towards the expression of the N-cadherin gene (an indicator of mesenchymal cells) causes a change in the cell matrix (1).

Since past studies have indicated that Hedgehog signaling alters the ability of cancer cells to attach to the epithelium and invade the extracellular matrix (25), the expression levels of Claudin-1 genes (an important gene involved in tight intercellular junctions), E-cadherin was investigated as an indicator of epithelial cell and MMP2 as a key metalloproteinase in the process of metastasis. As expected, a significant positive correlation between the expression levels of Smo and MMP2 genes was seen in tumor samples with advanced stages of metastasis. It is worth mentioning that the low expression levels of E-cadherin and Claudin-1 genes in tumor samples with advanced stages of metastasis were associated with the increased expression level of Smo.

Overall, the present study revealed a new level of molecular complexity that should be considered in managing invasive ductal carcinoma of the breast. Therefore, further identification of the pivotal role of the Hedgehog signaling pathway in invasive ductal carcinoma of the breast will reveal more facts in the field of breast cancer progression. In this regard, it seems that the overregulation of the Smo signaling receptor in the Hedgehog pathway and its inverse expression relationship with the critical gene of cell tight junction 1-Claudin may cause a poor prognosis in invasive ductal carcinoma of the breast. However, the clinical significance of this pathway requires more research.

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