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Original Article

RNA sequencing reveals the differential expression profiles of RNA in metastatic triple negative breast cancer and identifies SHISA3 as an efficient tumor suppressor gene

Noura Khaled[1,2], Nicolas Sonnier[2], Ioana Molnar[1,2], Flora Ponelle-Chachuat[2], Myriam Kossai[1,2], Nina Radosevic-Robin[1,2], Maud Privat[1,2], Yannick Bidet[1,2]

[1]Université Clermont Auvergne, Inserm, IMoST UMR 1240, F-63000 Clermont-Ferrand, France; [2]Centre Jean Perrin, Laboratoire d'Oncologie Moléculaire, IMoST UMR 1240, F-63000 Clermont-Ferrand, France

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Abstract: Breast cancer metastasis is the second leading cause of female mortality worldwide. Because of the heterogeneity within the group, metastatic biomarkers for triple-negative breast cancer (TNBC) providing predictive and prognosis values are urgently needed. Using RNA-Seq, we analyzed the transcriptome profiles of two groups of TNBCs tumors with or without distant metastasis. Whole transcriptome sequencing identified a set of genes implicated in TNBC metastasis with major roles in cell-cell adhesion, immune-modulation, and Wnt/β-catenin pathways. We further selected the SHISA3 gene and studied its biological significance through a series of *in vitro* and *in vivo* experiments. SHISA3 is a tumor suppressor gene, involved in several types of cancer. However, little is known concerning the role of SHISA3 in TNBC. Our *in vitro* and *in vivo* studies demonstrate that overexpression of SHISA3 inhibits TNBCs cell proliferation, metastasis and colony formation, and TNBC growth in xenografts. Mechanistically, SHISA3 inhibits TNBCs development and growth via downregulation of the epithelial-mesenchymal transition. Taken together, these results identified SHISA3 as a novel tumor suppressor gene in TNBC and suggest that SHISA3 could serve as a therapeutic target for TNBC patients.

Keywords: Triple-negative breast cancer TNBC, metastasis, SHISA3, prognosis, biomarker

Introduction

Breast cancer is one of the most common malignancies, ranking as high as the first leading cause of cancer mortality worldwide in women [1]. Over the years, advanced molecular and histological classifications, early detection, and personalized treatments contributed to a decrease in the mortality rate associated with breast cancer. These progresses revealed that a subtype of mammary tumors called "triple negative" remains of poor diagnosis [2]. Counting for about 15% of breast cancers, Triple Negative Breast Cancers (TNBC) are characterized by the lack of expression of estrogen receptor α (ERα) and progesterone receptor (PR) and no overexpression of human epidermal growth factor receptor 2 (HER2). These characteristics prevent them to benefit from effective targeted therapies [3]. Although chemotherapy is effective against these highly proliferative tumors, more than 40% evade to metastasis, and TNBCs are associated with the highest rate of relapse, and the worst survival outcomes [4]. Hence, elucidating molecular mechanisms involved in the process of invasion and metastasis of TNBC is urgently needed. New insights could come from the definition of TNBC deregulated metastasis-related genes. In return, the identification of novel molecular metastasis biomarkers and effective druggable targets would be important clinical breakthroughs to improve the prognosis of TNBC.

TNBC is recognized as a highly proliferative and invasive subtype. In order to evolve into metastases, primary tumors have to go through epithelial-mesenchymal transition (EMT) [5]. EMT

allows polarized immobile epithelial cells with tight cell-cell adherence to transform into individual motile mesenchymal cells acquiring exceptional migratory and invasive capacities [6]. This process is subtly regulated by several signalling pathways including TGFβ, Notch, Wnt, and most growth factor receptors. It has also been shown that epigenetic modifications are major regulators of the EMT [7]. Whatever the pathways involved, they converge on key transcription factors considered as the starting points of EMT. Such nuclear regulators include ZEB1 and ZEB2, TWIST, SLUG, and SNAIL. Their combined action switches the expression of structural proteins such as E- and N-Cadherin or Vimentin [6]. The activation of EMT is highly associated with the poor prognosis and overall survival of TNBC due to its role in tumor progression, therapy resistance and escape, tumor recurrence, and metastatic dissemination to distant organs [8-10]. Hence, reversing EMT is of great interest nowadays as a therapeutic target improving the treatment and care of TNBC patients.

As a member of Wnt pathway, SHISA3 has been investigated in several types of cancers, and it is now described as a tumor suppressor gene. SHISA3 has been revealed to behave as an antagonist of the Wnt/β-catenin pathway via promoting β-catenin degradation in lung cancer [11]. Moreover, SHISA3 was reported to be epigenetically silenced in colorectal and laryngeal cancers associated with poor prognosis [12, 13]. Recently, *Shahzad et al.* demonstrated that SHISA3 is inactivated by hypermethylation in breast cancer and that regaining SHISA3 function classifies it as a tumor suppressor gene in breast cancer [14]. However, little is known concerning the role of SHISA3 in TNBC.

In this study, we explored the RNA expression profiles of metastatic TNBC (mTNBC) and non-metastatic TNBC (non-mTNBC) using high-throughput RNA sequencing (RNA-seq). Gene enrichment analyses performed on 1309 significantly dysregulated RNAs in mTNBCs compared with non-mTNBCs tissues reveal potential pathways and networks implicated in TNBC progression and metastasis. This study points out the role of SHISA3 in TNBC as a tumor suppressor gene. SHISA3 is downregulated in TNBC cell lines and metastasis-prone tumors. Our *in vitro* experiments demonstrated that overexpressing SHISA3 inhibits TNBC cells proliferation, colony-forming, migration, and invasion, therefore, inhibiting TNBC metastasis. *In vivo* experiments on a mice model confirmed these results showing a significant reduction of tumor size.

Materials and methods

Patients and specimens

Inclusion criteria for this study were as follow: a) Metastasis-free state at diagnosis; b) Confirmation of all triple-negative cases by a single pathologist; c) Availability of a five-year follow-up post-diagnosis; d) Availability of tumor samples.

A total of 6,340 breast cancer patients were registered at the Jean Perrin Center JPC, Clermont-Ferrand, France between January 2001 and October 2014. Among them, 833 specimens of TNBC patients were identified, resulting in a 13.1% fraction of TNBC similar to the expected proportion. Due to limited quantities of RNA, only 26 TNBC samples were kept for whole transcriptome RNA-Seq. Clinicopathological parameters of patients in the training and validation cohort are presented in **Table 1**. All JPC patients were informed about the study and expressed their non-opposition to use their tumor sample. Samples were provided by Biological Resource Center of Jean Perrin Comprehensive Cancer Center (No. BB-0033-00075), accredited number AC-2018-3319.

RNA extraction and RNA sequencing

Total RNA was isolated from formalin fixed paraffin embedded (FFPE) tissues using AllPrep DNA/RNA Micro Kit (Qiagen, France), with DNAse treatment. The quality and quantity of total RNAs were determined using 2100 Bioanalyzer (Agilent Technologies). cDNA libraries were prepared using KAPA RNA HyperPrep Kit with RiboErase (Roche Diagnostics). This kit removes most of ribosomal RNA but keeps all other types of RNA. The procedure followed supplier recommendations, with 25 ng of total RNA input. Subsequent indexed RNA sequencing of cDNA libraries with single-end reads (1 × 75 bps reads) was performed according to the standard Illumina protocol using NextSeq 550 High Output v2 kit (Illumina). 15 Million reads

Table 1. Clinical summary of patient characteristics in JPC datasets

Variables		Overall (n=26)	mTNBC (n=10)	non-mTNBC (n=16)	*p*-value
Age (Mean ± SD)		53.0 ± 12.4	53.44	51.91	0.7959
Metastatic relapse within 5 years	Yes	0.384			
	No	0.616			
T stage	T1	0.5384	0.69	0.3	0.0178
	T2	0.2692	0.31	0.2	
	T3	0.1153	0	0.3	
	T4	0.0769	0	0.2	
N stage	N0	0.5384	0.75	0.2	0.0270
	N1	0.2692	0.125	0.5	
	N2	0.0769	0.125	0	
	N3	0.1153	0	0.3	
Histologic grade	1	0	0	0	0.3291
	2	0.423	0.5	0.3	
	3	0.5769	0.5	0.7	
Ki-67	≤50%	0.46153	0.44	0.4	0.4588
	> 50%	0.53846	0.56	0.6	
Infiltrating Ductal Carcinoma		0.923	1	0.8	0.1679
In situ Ductal Carcinoma		0.077	0	0.2	

minimum were generated per sample (average 42,8 M).

Bioinformatic analyses

Demultipexed reads were aligned to the human reference genome (GRCh37/hg19) using Star software (v2.7) with default parameters. HTSeq (v0.11.2) was used to quantify raw gene expression. R package DESeq2 software v1.26.0 was used to carry out data normalization for sequencing depth differences and differential expression analyses. Differentially expressed genes were identified based on a significant *p*-value (P<0.05).

Functional enrichment analysis of DEGs

To reveal the functions of DEGs, Gene Ontology GO annotation, Ingenuity Pathway Analysis IPA, and KEGG pathway enrichment analyses were conducted to obtain further insights into the function of the DEGs in the biological functions and pathways most related to TNBC. The GO terms were comprised of the following three divisions: biological process, cellular component and molecular function. Adj. P<0.00005 was regarded as statistically significant with a presentation for only the top 10. Enrichment analysis was applied to all 1309 DEGs.

Cell lines

The human triple negative breast cancer cell lines MDA-MB-231 and HCC1937 were purchased from the American Type Culture Collection (Manassas, VA, USA). Cell lines were cultured in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Gibco, NY, USA), 2 mM glutamine, and 20 μg/ml gentamicin (Invitrogen, Grand Island, NY, USA) and incubated in a humidified incubator (37°C) with 5% CO_2 atmosphere.

Plasmid and transfection

Full-length SHISA3 cDNA (NM_001080505.2) cloned in pcDNA3.1 (pSHISA3) was provided by Genscript (Leiden, the Netherlands), as well as the native plasmid (pControl) used as control. Transfections were performed with final concentrations of 0.05 ng/μl of SHISA3 vector using the Lipofectamine™ 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR)

Total mRNA was extracted from both cell lines using the mRNAeasy Kit (Qiagen, France) according to the manufacturer's instructions.

Complementary DNA was prepared using SuperScript IV Reverse Transcriptase (Thermofisher, Invitrogen, Waltham, Massachusetts, USA). SHISA3 expression was determined by quantitative real-time PCR using PrimeTime qPCR Probe Assay # Hs.PT.58.39641562 and PrimeTime Gene Expression MasterMix from Integrated DNA technologies IDT (Coralville, Iowa, USA). RealTime PCR was performed on the 7900HT Fast Real-Time PCR System (Applied Biosystems) with 50 ng of RNA. Conditions were 10 min at 95°C followed by 42 cycles composed of 15 s at 95°C and 1 min at 60°C.

Western blotting and antibodies

Protein extraction was realized using RIPA buffer containing a complete phosphatase (Sigma Aldrich P5726) and protease inhibitor cocktail (Sigma Aldrich P8340). Whole cell lysates were run through a Bradford protein concentration assay for protein quantification. 30 µg of proteins were loaded and separated onto 12.5% SDS-PAGE and gels were transferred onto nitrocellulose membranes (Merck Millipore). After blocking with 5% non-fat dry milk in 1× TBS-0.1% Tween-20, the membranes were probed with the specific primary antibodies in blocking solution at 4°C overnight (final dilution of 1:100 for Snail (Cell signaling), 1:200 for ZEB1 (Santa Cruz), 1:1000 for E-cadherin (Cell signaling), Vimentin (Abcam), phosphorylated and non-phosphorylated β-catenin (Cell signaling), and Slug (Cell signaling), and 1:2000 for ZEB2 (Invitrogen). After three washes for 5 min with TBS-0.1% Tween-20, blots were incubated for 1h30 at room temperature with horseradish peroxidase HRP-conjugated secondary antibody diluted at 1:2000 in blocking solution (α-actin (Santa Cruz), Tubilin (Invitrogen), GAPDH (Cell signaling)). Protein bands were then detected and analyzed by chemiluminescence using a ChemiDoc image analyser (BioRad, Richmond, CA, USA). Densitometric analyses were performed using ImageJ software program (National Institutes of Health, Bethesda, MD, USA).

Transwell invasion assay

Cell invasion was examined using 24-well coated Matrigel invasion Chambers (Cat NO. 354480, Corning, New York, United States). 80 000 MDA-MB-231 and 110 000 HCC1937 transfected cells diluted in serum-free RPMI-1640 medium were seeded in the upper chamber of each insert, while a 10% fetal bovine serum medium was placed in the lower chamber. After 27 hours of incubation, inserts were washed, cells remaining on the inner surface of the membrane in the upper insert were removed and cells invaded through the membrane to the bottom surface were fixed with 4% paraformaldehyde and then stained with 0.5% crystal violet. Five representative images of the membrane (representing 1/3 of the surface) were taken using an inverted microscope and migrated cells were counted using ImageJ software. All the assays were performed in triplicate.

Cell proliferation assay

Cell proliferation was quantified using Resazurin reagent as a metabolic indicator for living cells. 25 µg/ml of Resazurin in 1× RPMI medium without phenol red was added to 13 000 MDA-MB-231 or 17 000 HCC 1937 cells seeded and transfected in a 96-well plate. 24 hours after transfection, cell absorbency at 590 nm was measured in a microplate reader (Fluoroskan, Thermofischer) to determine cell viability.

Apoptosis analysis

Cell apoptosis was analyzed using the FITC Annexin V Apoptosis Detection Kit with Propidium Iodide (Biolegend, San Diego, CA) according to the manufacturer's instructions. 24 hours post transfection, MDA-MB-231, and HCC1937 cell lines were stained with FITC and Propidium Iodide followed by fluorescence-activated cell sorting (FACS) analysis (BD Biosciences). All the assays were performed three times.

Wound healing assay

Wound healing assay was used to assess cell migration after transfection. 2×10^5 and 4×10^5 cells per well were seeded in a six-well plate for MDA-MB-231 and HCC1937 respectively. 24 hours post transfection, the cell mat was scratched with a sterile pipette tip and washed with phosphate-buffered saline (PBS) before adding a complemented medium. The wound area was imaged every 2 hours for 48 hours using a ZEISS observer microscope and its size was measured using ImageJ software (NIH,

Bethesda, MD, USA). The percentage of closure was calculated as the wound area at 24 h divided by the wound area at 0 h, in each group.

Soft agar colony formation assay

In 6-well plates, 3 000 MDA-MB-231 cells and 5 000 HCC 1937 cells per well were plated in 0.3% Noble agar in 0.5× RPMI and they were overlaid by 0.5% Noble agar in 1× RPMI. Agar layers were overlaid with 200 µl RPMI medium supplemented with 10% FBS, gentamicin (20 µg/ml), and glutamin (2 mM). This medium layer was aspirated and replaced three times per week. After two weeks of growth, medium was aspirated, and cells were stained with 200 µl nitrotetrazoline blue chloride (NTB) overnight. Colonies were then imaged and counted using Image J software.

In vivo tumorigenesis

For tumorigenesis assay, TNBC cells were transfected with SHISA3 and control plasmids for 24 hours. 10 Millions of MDA-MB-231 and HCC1937 transfected cells diluted in PBS were injected in the mammary gland of eleven-week-old female nude mice NMRI-Foxn1 (Janvier laboratories, Saint Berthevin Cedex, France). Tumorigenicity and tumor growth were assessed by measuring mice weight and tumor dimensions with calipers for up to 33 days (7 mice per group).

All animal studies were carried out in accordance with directive 2010/63/EU and under evaluation and approval of the institutional review board (CEEA, MENESR, authorization APAFIS#23214-2020011014348971 v4). All the mice were euthanized and dissected 33 days after injection.

Statistical assay

Statistical analyses were performed using R software (R-project, v.3.6.1, GNU GPL). The hypothesis tests were two-sided with 5% significance level. Correlation between SHISA3 expression level and clinico-pathological characteristics was investigated using Spearman's correlation coefficient, Welch's t-test and Kruskal-Wallis test. We analyzed the influence of SHISA3 on proliferative rates and on wound closure/Transwell migration using linear mixed models (replicate as random effect), on cell apoptosis by chi-squared test of independence, and on colony-forming ability by Student's t-test. Comparison of tumour volume evolution between the two conditions was tested using linear mixed model (subject as random effect, condition × time interaction term), and post-hoc Šidák-adjusted comparisons.

Results

Transcriptome analyses of mTNBCs and non-mTNBCs

To highlight the differences between the tumors that develop into metastases (mTNBC) and those that do not (non-mTNBC), we carried a retrospective study enrolling patients according to their metastatic status. Proof of non-relapse is gained by a certified 5-years+ follow up. 26 patients met our inclusion criteria, divided into 16 non-mTNBC and 10 mTNBC. This distribution (38,5% of relapse) is consistent with global clinical observations and rules out a major recruitment bias. The clinicopathologic features of the 26 patients with TNBC are summarized in **Table 1**. Whole transcriptome RNA sequencing was performed on the corresponding FFPE samples. A total of 46 426 distinct RNAs were detected and quantified. Data were normalized and differentially expressed RNAs were revealed by fold-change filtering ($|\log2(\text{fold change})| > 1$) and Student's t-testing (p-value <0.05). 1309 differentially expressed genes (DEGs) were identified in mTNBCs relative to non-mTNBCs patients. Among them, 741 were downregulated and 568 were upregulated in mTNBCs. The top 10 upregulated and 10 downregulated genes in mTNBCs are presented in **Table 2**.

To further examine the role of the DEGs in the metastatic mechanism of TNBC, functional enrichment analyses of the 1309 differentially expressed genes were performed using different bioinformatic tools such as Ingenuity pathway analysis (IPA), KOBAS (KEGG Orthology Based Annotation System) and Gene Ontology (GO) powered by PANTHER. IPA revealed the top 5 molecular and cellular functions associated with the DEGs in mTNBCs. A p-value was calculated considering the number of molecules involved in each function and "cellular development", "growth and proliferation", "morphology", "death, and survival" along with "cell-to-cell signaling and interaction" are significantly enriched in mTNBC (**Figure 1A**). Our analysis

Table 2. Most misregulated RNAs in mTNBCs vs non-mTNBCs

	log2FoldChange	Padj	Regulation
CDH19	-6.338559637	5.06771E-06	Down
LCN8	-5.871648287	0.019340481	Down
COL2A1	-5.592419049	3.01272E-05	Down
IGHV1-45	-5.498887485	0.01157546	Down
RP5-887A10,1	-5.121944635	0.025136582	Down
RP11-927P21,2	-5.121072061	0.000963901	Down
CMTM5	-5.089100867	0.032019477	Down
RP4-601P9,2	-5.030837943	0.009260712	Down
AC105402,4	-4.903065775	0.007790738	Down
CR2	-4.828943604	0.001168588	Down
TCN1	11.17243743	0.009346551	Up
LINC01511	9.495083743	0.000181268	Up
DHRS2	8.644242252	9.96015E-15	Up
S100A7A	8.186221784	2.45844E-08	Up
PADI3	8.007493764	8.11258E-12	Up
UGT2B28	7.914714995	0.002934248	Up
SPINK8	7.594703075	0.002440415	Up
HSD3BP2	7.272657702	0.014425552	Up
S100A7	7.269045169	1.19712E-08	Up
PNLIPRP3	6.908248743	0.015976542	Up

further revealed a highly significant overlap of 10 canonical pathways (P<0.005) with KEGG connected with metabolic pathways, T cell receptor signaling pathway, and cell adhesion molecules (CAMs) presented in **Figure 1B**. In addition, the same DEGs in mTNBCs were analyzed with Gene Ontology classifying in three major domains: cellular component, molecular function, and biological process. The top 10 enriched GO of each domain are presented in **Figure 1C**. Cell surface receptor signaling pathway, Notch binding, and transcription regulator complex are the most significant enriched GO terms in the biological process, molecular function, and cellular component, respectively.

Cross analyzing these three enrichment experiments revealed that general signaling pathways were significantly enriched between our two groups of patients including cellular morphology, cell to cell signaling, and pathways in cancer. Our attention was caught by one in particular. Gene Ontology depicts three enriched biological processes, two molecular functions and one cellular component related to Wnt pathway. This dramatically enriched pathway fits IPA and KEGG results, and it is also known as an essential EMT-regulator. The choice of a candidate gene of the Wnt pathway was based on both high differential expression and literature. SHISA3 was underexpressed in metastasized tumors with a highly ranked *p*-value. It is among the top 50 downregulated genes in mTNBCs with log fold change = -3.5 and *p*-value adj = 0.01. Moreover, although SHISA3 was mentioned as a tumor suppressor gene in different types of cancer such as leukemia, colorectal cancers and others, the literature shows that there is no evidence on the role of SHISA3 in CSTNs. Combining a putative role but a lack of evidence, high differential expression and involvement in an enriched pathway, we therefore selected SHISA3 as our candidate gene to investigate its role in vitro and in vivo on the metastasis of TNBC. The expression of SHISA3 is significantly downregulated in human tumors compared to their corresponding normal tissues (data not shown). This suppression of expression is confirmed in breast tumors relative to normal tissue (**Figure 2A**).

Furthermore, we evaluated the correlation between SHISA3 expression level and the clinical or pathological characteristics in the two groups of patients with or without metastasis (cutoff value of median). Due to our low headcount, no statistically significant relationships were reported between SHISA3 negative expression and different clinical characteristics including age, histologic grade, proliferation factor KI67, N stage, TNM stage, and 5-years metastatic relapse (data not shown).

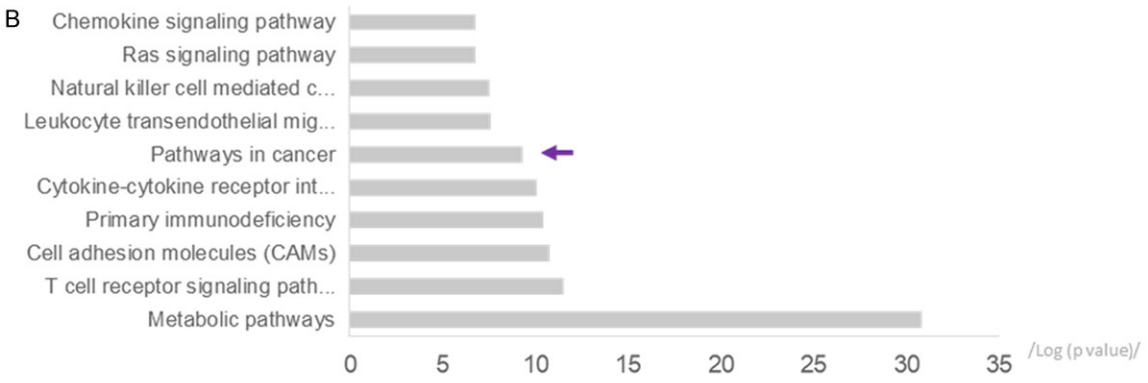
SHISA3 overexpression decreases TNBC cell lines aggressivity in vitro

Given that significantly low SHISA3 expression was revealed in metastatic TNBCs, we investigated whether SHISA3 can regulate TNBC phenotypes. First, we analyzed SHISA3 expression in various normal tissues and triple-negative breast cancer cell lines. Data from GTEx and CBio Portals showed that, although SHISA3 is expressed at low level in normal breast, variability exists among invasive triple-negative

A

	P-value range	# Molecules
Cellular development	7,56E-07 - 2,90E-32	141
Cellular Growth and Proliferation	7,56E-07 - 2,90E-32	140
Cellular Morphology ←	6,86E-07 - 1,74E-29	75
Cellular Death and Survival	3,91E-07 - 2,43E-28	224
Cell-to cell Signaling and Interaction ←	4,65E-07 - 2,52E-27	151

B



C

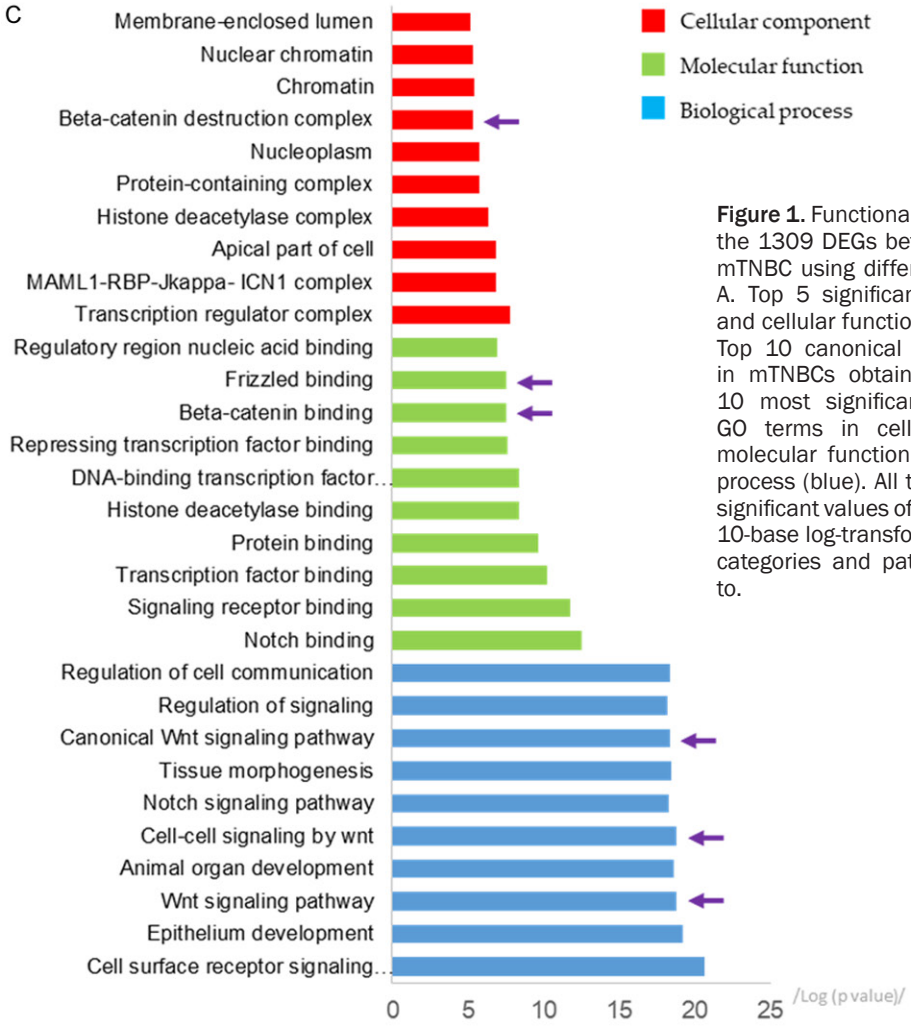


Figure 1. Functional enrichment analyses of the 1309 DEGs between mTNBC and non-mTNBC using different bioinformatic tools. A. Top 5 significantly enriched molecular and cellular functions in mTNBCs by IPA. B. Top 10 canonical pathways misregulated in mTNBCs obtained with KEGG. C. Top 10 most significantly ($P<0.05$) enriched GO terms in cellular component (red), molecular function (green), and biological process (blue). All the adjusted statistically significant values of GO terms were negative 10-base log-transformed. Blue arrows point categories and pathways SHISA3 belongs to.

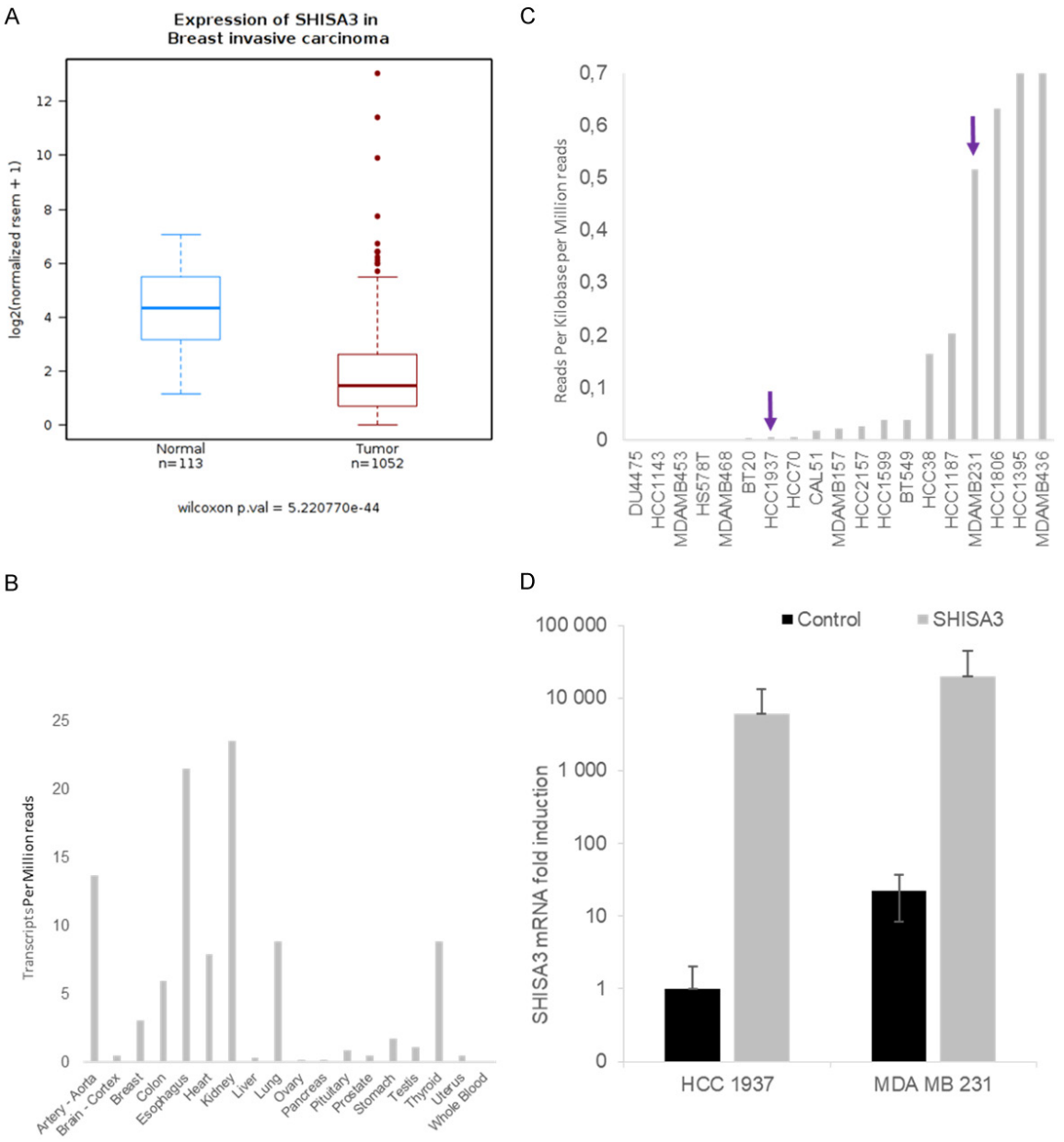


Figure 2. Expression of SHISA3 in human triple negative breast cancer TNBC. A. SHISA3 mRNA level was downregulated in breast invasive carcinomas relative to normal tissues *(TCGA Wanderer).* B. Expression of SHISA3 mRNA across various normal tissues. C. Expression of SHISA3 mRNA across various breast cancer cell lines. Blue arrows show selected cell lines. D. qRT-PCR was used to detect SHISA3 overexpression efficiency in HCC 1937 and MDA MB 231 cells.

breast cancer cell lines (**Figure 2B** and **2C**). For further overexpression experiments, we selected two cell lines with different SHISA3 basal expression: MDA MB 231 is an example of "high" expression within breast cells and HCC 1937 expresses SHISA3 at a low-but-not-null level. Transfection of a plasmid expressing the full-length cDNA of SHISA3 into both cell lines revealed a robust over-expression of SHISA3 detected by qPCR (**Figure 2D**).

Several polyclonal antibodies against SHISA3 were tested and nonerevealed to be specific. Some are new productions of published references, but with a different affinity (data not shown). Due to the lack of specificity of SHISA3 antibodies on market today, SHISA3 overexpression could not be validated at the protein level. We further evaluate the influence of SHISA3 overexpression on cell proliferation, colony formation ability, apoptosis, invasive-

ness, and migration. Enhanced cell proliferation is one of the key hallmarks of malignant transformation. In this context, we evaluated the effect of SHISA3 overexpression on the proliferative ability of TNBC cell lines using the cell viability Resazurin assay. The proliferative rates of HCC 1937 and MDA MB 231 overexpressing SHISA3 were slightly reduced relative to the corresponding control cell lines ($P \leq 0.005$ for both cell lines) (**Figure 3A**). This result shows that TNBC cells overexpressing SHISA3 tend to have a lower proliferative capacity than controls. To further explore the role of SHISA3 in tumorigenesis, we measured the apoptosis level in TNBC cell lines overexpressing SHISA3. Flow cytometry analysis of annexin V divided cells into three categories consisting of early apoptosis, late apoptosis or necrosis, and living cells. Our results indicate that SHISA3 does not affect TNBC cell apoptosis compared to the control (**Figure 3B**). We have further explored the role of SHISA3 in TNBC aggressivity with soft agar colony formation tests in order to evaluate the anchorage-independent growth ability of SHISA3 overexpressing cells. The clonogenic assay revealed that SHISA3 significantly inhibits the colony-forming ability of HCC1937 cell line ($P \leq 0.05$) in comparison with the control vector cells while no statistically significant result was shown to MDA MB 231 cell line ($P=0.14$) (**Figure 3C**).

Given that SHISA3 was suppressed in patients with metastatic TNBCs, we next investigated the effects of SHISA3 overexpression on migration in HCC 1937 and MDA MB 231 cells, with awound-healing assay. The experiment showed a significantly smaller wound closure in SHISA3 overexpressing HCC1937 ($P \leq 0.05$) and MDA MB 231 ($P \leq 0.005$) cell lines than the cells with an empty vector (**Figure 3D**). These results indicate that SHISA3 inhibits TNBC cell migration *in vitro*. In consistent with these results, the invasive potential of TNBC cells with SHISA3 overexpression was addressed with a Transwell matrigel assay. A significant decrease in the number of invaded TNBC cancer cells was demonstrated after SHISA3 overexpression relative to cells without SHISA3 overexpression with $P \leq 0.005$ for HCC1937 cell line and $P \leq 0.05$ for MDA MB 231. Data are presented in **Figure 3E**. These results indicate that SHISA3 inhibits the invasion of TNBC cancer cells *in vitro*.

Taken together, SHISA3 inhibits the growth (dependant or not of anchorage), migration, and invasion of TNBC cells *in vitro* with no effect on the apoptosis capacity.

SHISA3 expression tend to blocks EMT

Accumulating evidence suggests that EMT, a process by which epithelial cells undergo transdifferenciation into motile mesenchymal cells, plays a critical role in TNBC [15]. In order to investigate whether SHISA3 regulates EMT, we transiently expressed SHISA3 in the two highly invasive cell lines HCC 1937 and MDA MB 231. As shown in **Figure 4A**, increased expression of SHISA3 up-regulated epithelial markers like E-cadherin in HCC1937, whereas mesenchymal markers such as Vimentin were shown to be slightly down-regulated in MDA MB 231. Both markers were not co-expressed in these cell lines, and neither is N-cadherin. Transcription factors such as ZEB1, ZEB2, SLUG, and SNAIL1, whose high expression was considered a hallmark of EMT, were also studied [15]. In HCC 1937 overexpressing SHISA3, SLUG, SNAIL, and ZEB1 were slightly downregulated while ZEB2 was not expressed. SLUG and SNAIL showed downregulation in MDA MB 231 cells with SHISA3 overexpression with no effect on ZEB1 and ZEB2 (**Figure 4A**, **4B**). Consistently, overexpression of SHISA3 appears to participate in the slow-down of EMT progression in both TNBC cell lines HCC1937 and MDA MB 231. The mechanism of action seems to rely mainly on SNAIL and SLUG and less on ZEB factors.

Taking advantage of the enrichment analyses suggesting a role of the Wnt signaling pathway in metastasis formation in TNBC, we examined the expression of β-catenin when SHISA3 is overexpressed. Western Blot analyses showed that both active and inactive forms of β-catenin are reduced in HCC1937 but not in MDA MB 231 in cells overexpressing SHISA3 (**Figure 4**). Hence, β-catenin might be a target of SHISA3 regulating thereby Wnt pathway in some TNBC cell types. However further studies are needed to explore other targets for SHISA3.

Overexpression of SHISA3 inhibits TNBC progression in vivo

To explore the effects of SHISA3 on TNBC tumorigenesis *in vivo*, SHISA3 overexpressing HCC 1937 and MDA MB 231 cell lines were

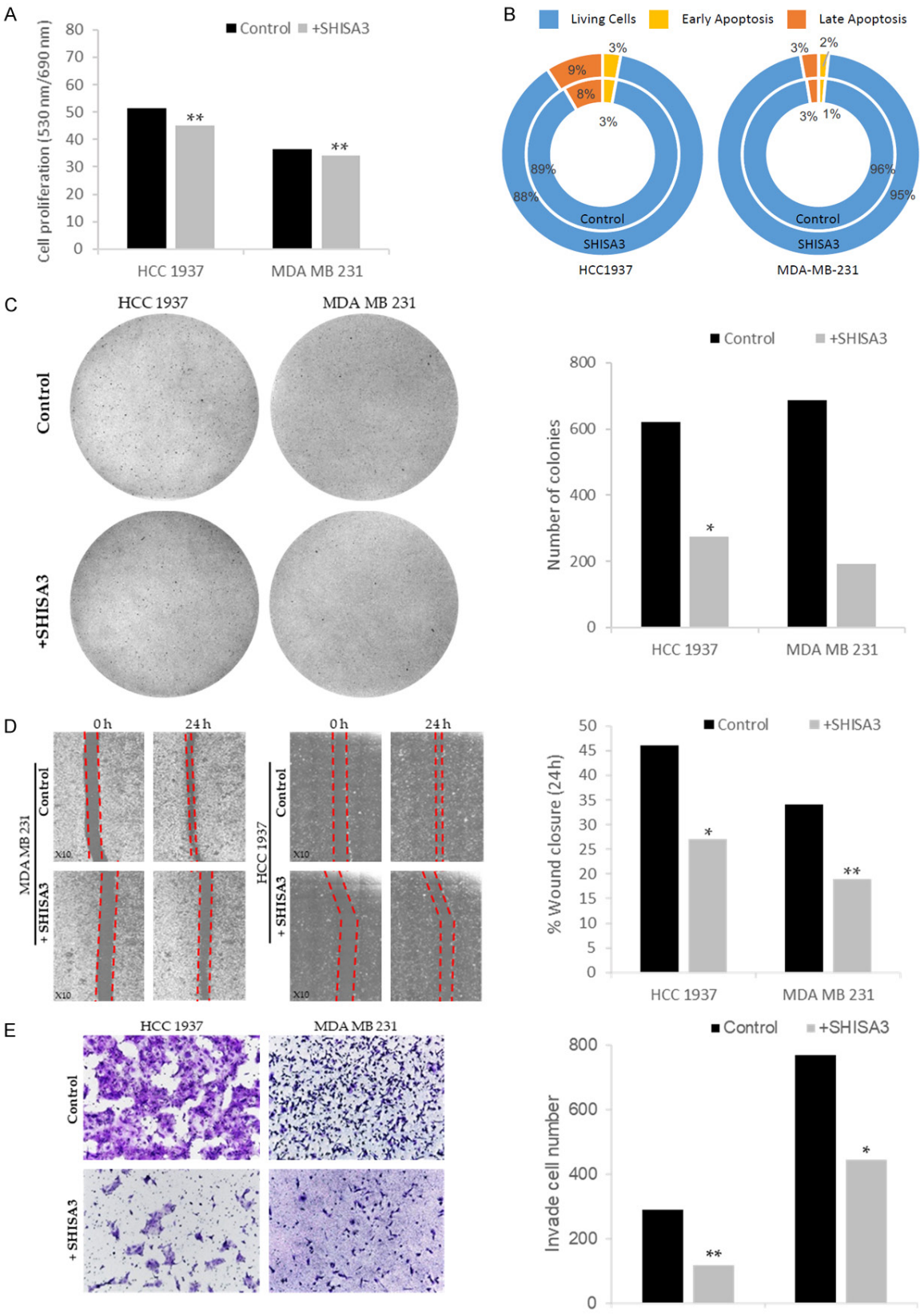
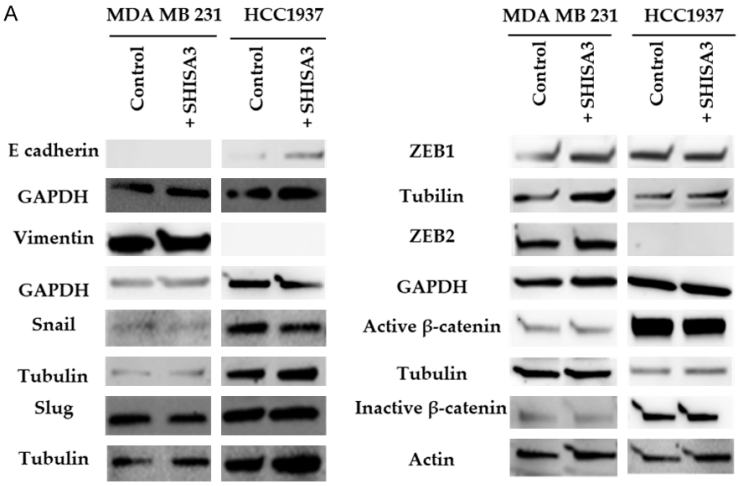


Figure 3. Overexpression of SHISA3 in triple-negative breast cancer cell lines decreases agressivity. All experiments were conducted three times independently. Error bars represent the mean ± SD. *P≤0.05 AND **P≤0.005. A. Cell growth rate from parental and SHISA3 upregulation HCC 1937 cells (left) and MDA MB 231 cells (right) at 24 h post-

transfection were determined by Resazurin cell proliferation assay. B. Apoptosis was assayed by flow cytometry after cell labelling with Propidium Iodide (IPA) and Annexin V coupled with FITC. Inner circles of the graph represent the apoptosis state of cells transfected with a control plasmid. Outer circles represent the apoptosis state of cells transfected with SHISA3. C. Anchorage-independent colony formation on soft agar assay for HCC 1937 and MDA MB 231 cells. D. Wound healing assays were performed to investigate the role of SHISA3 in HCC 1937 and MDA MB 231 cell migration. Observations by Zeiss microscopy were recorded at 0 and 24 h after the scratch of the cell surface. The panel shows the quantitative analysis of the wound closure of the empty vector and SHISA3 overexpressing cells. E. Transwell assays were performed to explore the role of SHISA3 in HCC 1937 and MDA MB 231 cell invasion. The images were obtained 24 h after seeding. The number of cells was counted in five representative fields per Transwell (×10). Quantitative analysis of the number of invasive cells is presented in the corresponding panel.

A



B

	MDA MB 231		HCC 1937	
	Control	+ SHISA3	Control	+ SHISA3
E cadherin	__	__	0.25	1.28
Vimentin	1.06	0.87	__	__
Snail	4.50	1.34	1.24	0.80
Slug	2.26	1.68	1.50	1.08
ZEB1	0.99	1.00	1.84	1.26
ZEB2	0.70	0.65	__	__
β-catenin	0.75	0.76	2.58	1.61
℗-β-catenin	0.74	0.46	1.30	0.55

Figure 4. SHISA3 participates in the repression of EMT markers and Wnt signaling pathway in triple-negative breast cancer cells. A. Western blotting analysis of EMT markers and transcription factors in HCC1937 and MDA MB 231 cell lines with SHISA3 overexpression. Phosphorylated β-catenin is the inactive form, targeted to degradation. B. Quantification of EMT markers according to their control. ImageJ was used for quantification.

orthotopically injected into the fourth mammary fat pad of NMRI Nude Fox n1 nude mice, and the tumor volumes were measured weekly for 33 days. No tumor has developed in mice injected with HCC 1937 cell lines. Results in **Figure 5** represent the sizes of the tumors generated with the MDA MB 231 cells. Tumor volume was significantly smaller in the group overexpressing SHISA3 at day 33, just before sacrifice (P=0.01). In addition, the evolution of the tumor volume over time was significantly reduced in the the group overexpressing SHISA3 (P<0.001).

Discussion

To our knowledge, our work is the first study performing RNA sequencing to reveal differentially expressed genes in mTNBCs relative to non-mTNBCs tissues. mTNBCs and non-mTNBCs patients were carefully selected with a triple-negative histological profile and a follow-up of five-year post-diagnosis to classify patients in the two described groups. The small but well clinically defined dataset of JPC patients included in this study presents an important added value to the study. Indeed, a specific selection of included patients generated a rigorous and powerful list of DEGs. DEGs identified were mainly implicated in cancer cell proliferation, motility, development, and death along with cell-to-cell interaction, cell surface receptor signaling, and Wnt pathway.

Based on significant *p*-value, literature, and enrichment analyses, we selected SHISA3 gene, a repressed RNA in mTNBCs, to investigate its regulatory role in TNBC tumor progression and metastasis. SHISA3 is an endoplasmic reticulum-resident protein described to modulate both Wnt and FGF signaling. The best described regulatory role for SHISA3 is the inhibition of the post-translational maturation and cell surface trafficking of Frizzle and FGF receptors [16-18]. Although less explored, human SHISA3 was initially described as a tumor suppressor gene-

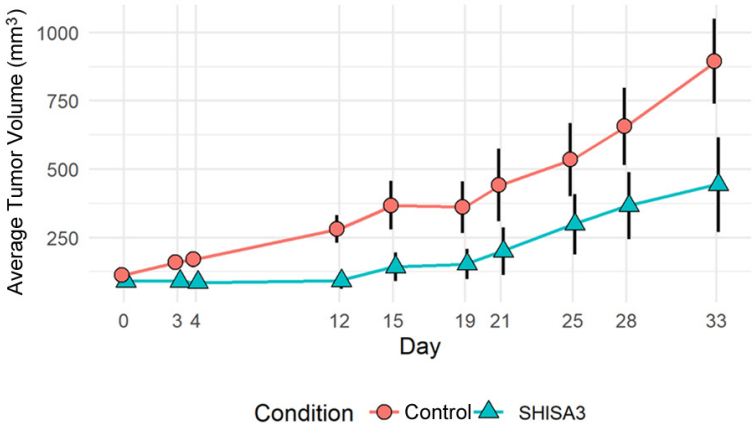


Figure 5. SHISA3 prevents tumor growth in vivo. The sizes of the tumors in the SHISA3 overexpressing group were significantly smaller than those of the control group at Day 33 *(P<0.01)*. Moreover, growth rate is significantly reduced over the period *(P<0.001)*.

regulating lung cancer metastasis via Wnt/β catenin signaling [11]. Later investigations revealed that SHISA3 is a novel tumor suppressor gene that is suppressed in several human malignancies. Recently, *Shahzad et al.* demonstrated that SHISA3 is epigenetically silenced in breast cancers and that SHISA3 ectopic expression reduced BC cell lines proliferation and migration [14]. However, the role of SHISA3 and the mechanism of action in TNBC remains largely unknown. Our study aims to investigate the role of SHISA3 in the metastasis specifically in TNBC, *in vitro* and *in vivo*. As expected, SHISA3 overexpression tends to reduce TNBC proliferation, with an inhibition of clonogenic forming ability, migration, and invasion *in vitro* as well as tumor growth *in vivo*. These findings are consistent with the previous studies presenting SHISA3 as a key regulator in inhibiting tumor cell migration and aggressiveness in different types of cancers including BCs.

Recent studies suggested that DNA hypermethylation of SHISA3 is the mechanism of gene inactivation responsible for the frequent suppression of SHISA3 in multiple types of tumors [13, 19, 20]. *Shahzad et al.* demonstrated that SHISA3 demethylation inhibits breast cancer proliferation and migration [14]. Hypermethylation of SHISA3 inducing a high metastatic ability in mTNBCs comparing to non-mTNBCs could be a strong hypothesis to investigate, along with further studies targeting SHISA3 with demethylating agents to investigate its influence on distant metastasis outcome. Metastasis is a pivotal mechanism associated with TNBC poor outcome and high mortality [21, 22]. EMT is the key metastasis phenomenon favoring the formation of distant metastasis promoting the malignant development of human malignancies [5]. Moreover, multiple signaling pathways were demonstrated to cooperate with EMT transcription factors affecting cell-cell adhesion and hence tumor cell migration [15, 23]. Although the molecular mechanisms explaining how SHISA3 acts as a metastasis regulator during TNBC progression have not been fully elucidated, previous SHISA3 research suggests a blocage of FZRD receptors contributing to β catenin degradation and inhibition of TCF/LEF complex [11]. Based on these researches, we thought to confirm the role of SHISA3 as a regulator of Wnt pathway in TNBC. Our results suggested that SHISA3 may act *via* inhibiting Wnt/β catenin pathway supporting thereby the work of *Shahzad et al.* [14]. Upcoming studies validating these suggestions in TNBC are needed. These results together shed the light on the pivotal role of SHISA3 in inhibiting TNBCs cell migration and invasion, regulating TNBCs development and metastasis.

Our findings reveal a lower level of β-catenin in SHISA3-positive TNBC cell lines. Literature has also described a role of Wnt pathway and β-catenin complex in several human malignancies including breast cancers [24, 25]. Moreover, Wnt signaling pathway is also known as a major modulator for the EMT process in several different cancer types [23, 26-28]. Aberrant canonical and non-canonical pathways Wnt signaling is a characteristic of TNBC tumorigenesis and metastasis [29]. Researchers have demonstrated an enrichment of Wnt/β-catenin signaling in TNBC with a strong association with poor clinical outcomes [30, 31]. TNBC patients displaying dysregulated Wnt/β-catenin signaling are more likely to develop lung and brain secondary metastases [32]. Moreover, *in vitro* studies reported an increase in cell migration, colony formation, stem-like features, and chemoresistance in TNBC cells with nuclear accu-

mulation of β-catenin [30]. Along with this, the accumulation of β-catenin promotes TNBC tumorigenesis in mouse cancer models [33]. Moreover, Wnt/β-catenin promotes not only the stabilization of β-catenin in the cytoplasm but also can affect a subsequent transcription of genes involved in EMT [34, 35]. This is consistent with our results of EMT-associated transcription factors ZEB1, ZEB2, SNAIL and SLUG, and epithelial and mesenchymal markers. SHISA3-TNBC cell lines present a higher level of E-cadherin and a low level of ZEB1, ZEB2, SLUG, and SNAIL. These results show that SHISA3 plays a role in reducing EMT in TNBC.

Conclusion

This study identified SHISA3 as a tumor and metastasis suppressor gene in TNBC and a valuable therapeutic target for metastatic TNBC in the years to come. Low expression of SHAISA3 contributes to tumor aggressivity. This could be done at least partly by modulating the canonical Wnt signaling pathway. Development of targeted therapies able to restore SHISA3 expression appears as a promising approach for the most aggressive TNBC.

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Disclosure of conflict of interest

None.

Address correspondence to: Yannick Bidet, Université Clermont Auvergne, Inserm, IMoST UMR 1240, F-63000 Clermont-Ferrand, France. E-mail: Yannick.Bidet@uca.fr

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