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Metastatic breast tumors downregulate miR-145 regulating the hypoxia-induced vasculogenic mimicry

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ABSTRACT

Tumor cells grow in three-dimensional (3D) channels-like structures denoted as vasculogenic mimicry (VM), which provides a route for nutrients and oxygen acquisition. VM is activated by hypoxia and associated with metastasis and poor prognosis. MetastamiRs are microRNAs regulating metastasis, however, if they control VM in breast cancer remains poorly understood. The aim of this study was to evaluate the expression of VM-associated microRNAs in tumors of metastatic breast cancer patients. Firstly, we constructed microRNAs/mRNAs co-regulation networks using expression data from TCGA databases. Dozens of microRNAs regulating genes involved in VM and metastasis were found. Of these, we selected 10 microRNAs for further characterization. The presence of VM in histological samples from patients with or without metastasis was evaluated using CD31-/PAS+ immunophenotyping. Remarkably, data showed that VM was significantly increased in tumors from patients with metastasis in comparison with no-metastatic group. Gene expression analysis indicated that miR-145, miR-142-3p, miR-31, miR-148a, miR-200b-3p and miR-526b were downregulated in primary tumors from patients with metastatic disease and positive for VM. Moreover, modulated microRNAs showed a predictive clinical value in overall survival in a cohort (n=1262) of breast cancer patients. Of these, we evaluated the role of miR-145 in formation of hypoxia-induced 3D channels-like using an *in vitro* model that recapitulates the early stages of VM. Data showed that miR-145 mimics was able to abolish the VM development in both metastatic Hs578t and MDA-MB-231 breast cancer cells. In conclusion, manipulation of miR-145 levels may represent a therapeutic approach in metastatic breast cancer patients that developed VM.

Introduction

Cancer cells needs extra supplies of nutrients and oxygen to support exacerbated cell proliferation. Angiogenesis, the formation of blood vessels from an existing vascular network, and vessel co-option are the classical cancer hallmarks associated with uptake of nutrients by tumors [1,2]. In consequence, diverse anti-angiogenic therapies have been developed for solid human tumors, although the clinical response remains limited due to the activation of the mechanisms of drug resistance, a phenomenon observed mainly in aggressive metastatic tumors, suggesting that alternative processes for tumor nutrition maybe occurring through unknown non-angiogenic pathways [3]. Recently, a novel phenomenon denoted as vasculogenic mimicry (VM) or vascular mimicry was discovered in aggressive melanoma and then confirmed in diverse solid tumors, emerging as an alternative route to fuel tumor growth [4]. VM refers to the ability of highly invasive tumor cells to

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form extracellular matrix (ECM)-rich channel-like networks that are fluid-conducting vessels with a hemodynamics resembling those occurring in classical blood vessels [5,6]. ECM-rich VM networks have been found in clinical tumor samples obtained from cancer patients, and have been associated with increased metastasis and poor prognosis [7–10]. Therefore, VM is considered as a tumor biomarker with prognostic value, as well as a novel potential therapeutic target in human cancers. Experimentally, VM could be recapitulated using diverse models based on the grown of cancer cells lines over three-dimensional (3D) matrices under a hypoxic microenvironment *in vitro* which allows the dissection of the molecular pathways and gene expression mechanisms of the phenomenon [11].

Metastasis is one of the main barriers for successful anti-cancer therapies, and responsible for a myriad of cancer-related deaths [12]. Molecular mechanisms of metastasis have been well documented including the prominent roles of non-coding RNAs (ncRNAs) indicating that the cellular process regulating disseminated disease are complex and intricated [13]. MetastamiRs are microRNAs involved in the post-transcriptional regulation of key protein-encoding genes involved in metastasis [14]. MicroRNAs are small ncRNAs of 21-25 nucleotides length which negatively regulate the gene expression by direct binding to multiple messenger RNAs (mRNAs) targets inducing its degradation by activation of the RNA interference pathway, and/or by inhibition of the mRNA's translation [15]. Moreover, recent findings indicate that long non-coding RNAs and microRNAs can regulate the VM *in vitro* and *in vivo*, suggesting that they could represent attractive molecular tools for personalized cancer therapies [16]. However, the role of microRNAs in the regulation of VM in metastatic disease remains poorly understood. Inspired by these facts, here we analyzed the presence of VM in a small set of breast tumors, and then evaluated the role of a selected microRNAs in the regulation of VM using an *in vitro* cell model. Our data indicates that miR-145 was downregulated in metastatic tumors and plays a function in VM formation. Implications of our finding for breast cancer biology and therapy are discussed.

Material and methods

Cell lines

Metastatic MDA-MB-231 and Hs-578t triple negative breast cancer cell lines were obtained from the American Type Culture Collection. Commercial cancer cell lines were used at passage 1 and routinely grown and maintained in Dulbecco's modification of Eagle's minimal medium (DMEM) supplemented with 10% fetal bovine serum, and penicillin-streptomycin (50 unit/mL; Invitrogen, Carlsbad, CA, USA) at 37°C in an incubator with 5% CO_2 atmosphere.

Clinical characteristics of breast cancer patients

Tumor samples were collected between 2020 and 2021 from 10 triple negative breast cancer patients after signed agreement. 5 cases of metastasis and 5 cases without metastases were included in the cohort. Formalin-fixed paraffin-embedded (FFPE) samples were collected from 10 patients with breast cancer who underwent surgery. The selection criteria for the samples were ductal carcinoma, triple negative subtype, stage II onwards, with the presence or absence of metastases. The present study was approved by the Ethics Committee of the Hospital Regional de Alta Especialidad Ixtapaluca (number NR-16-2020). Tissues were used where the pathologist confirmed the existence of at least 80% tumor cells in the clinical samples.

CD31/periodic acid Schiff (PAS) double staining

Sections of 3μm were stained using endothelial marker CD31 monoclonal antibodies (Bio SB clone 1A10). The tumor sections were deparaffinated in xylene and rehydrated with decreasing ethanol gradient, and heated with citrate buffer (pH=6) was used for the antigen retrieval during 30 min. The endogenous peroxidase was blocked with 3% hydrogen peroxide in 50% ethanol during 30 min. After, the nonspecific binding sites were blocked by 1% pig serum (Sigma). The anti-CD31 antibodies were used in 1:200 dilution, which was incubated by 1h to 37°C and after overnight to 4°C. Then, sections were washed with PBS1X and PBS 1X-Tween 20 and incubated with secondary biotinylated-antibody for 1 h to 37°C, followed by incubation with the HRP complex for 30 min (VECTASTAIN Elite ABC system). Histological samples were revealed with DAB, and reaction was stopped with water. Then, sections were incubated with 0.5% periodic acid solution and rinsed with distilled water. After that, samples were treated with Schiff reagent for 10 minutes and water rinsing. The sections were counter-stained with Harris hematoxylin, observed by optical microscopy and scanned with Aperio ImageScope - Software (Leica). Blood vessels and channels were counted using ImageJ Fiji software for IHQ DAB

RNA isolation from FFPE

RNA was isolated from formalin-fixed paraffin-embedded (FFPE) blocks using the RNeasy FFPE kit (Qiagen Inc, Valencia, California) following the manufacture's protocol. Briefly, 10 sections of 10 μm paraffin embedded samples were incubated in xylene for 1 h at 63°C for deparaffinization. Xylene was removed and 1 ml of absolute ethanol was added, mixed and centrifuged at maximum speed for 2 min. Ethanol was removed and samples were air dried. Total RNA was extracted using Trizol protocol (Ambion, Austin, Texas). RNA concentration and purity were evaluated for spectrophotometry (Nano-Drop Technologies, Wilmington, Delaware) and integrity was analyzed by 1% agarose gel electrophoresis.

Quantitative RT-PCR stem-loop TaqMan microRNAs assays

Quantitative RT-PCR analyses for the 10 microRNAs were performed using stem-loop TaqMan microRNA assays (Thermo-Fisher, Waltham, Massachusetts).100 ng total RNA was reverse transcribed using a specific stem looped-RT specific primer, dNTPs (100 mM), 1.0 μL reverse transcriptase MultiScribe (50 U/μL), 10× buffer, RNase inhibitor (20 U/μL), and RNase-free water. Then, retrotranscription reaction (1:15 dilution) was mixed with 10 μL master mix TaqMan Universal PCR Master Mix, No AmpErase UNG 2×, 7.67 μL RNase free water, and 1.0 μL PCR probe. Polymerase chain reaction was performed in a GeneAmp System 9700 (Applied Biosystems, Foster, California) as follows: 95°C for 10 minutes, and 40 cycles at 95°C for 15 s, and 60°C for 1 min. Tests were normalized using RNU44 as internal control. Experiments were performed by triplicate and results were expressed as mean ±S.D.

Vasculogenic mimicry assays

Vasculogenic mimicry experiments were performed using 3D cultures of breast cancer cells grown over commercial Geltrex matrix. Triple negative MDA-MB-231 and Hs-578t cells (1×10^4 cells/well) were transfected with miR-145 mimics (60 nM) or scramble (30 nM) as negative control. Cells were cultured in a 96-well plate covered with 50 μL Geltrex, incubated at 37°C in 5% CO_2 atmosphere under hypoxia conditions (1% O_2), for 48 h. Formation of 3D channel-like networks were quantified by counting under an inverted microscope (Iroscope SI-PH) for 0, 3, 6 and 9 h. Data were expressed as mean ±S.D.

Target genes predictions

Target genes of differential expressed microRNAs were predicted using miRTarBase portal (https://mirtarbase.cuhk.edu.cn/~miRTarBase/miRTarBase_2022/php/index.php) and TargetScanHuman 8.0 (https://www.targetscan.org/vert_80/). Experimentally validated microRNA-target interactions were selected with the aim to obtain

promising experimental evidence and to avoid overestimating the functions of deregulated microRNAs.

Analysis of signaling pathways and cellular processes

The DAVID database (http://david.abcc.ncifcrf.gov/) was used to perform gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of differentially expressed miRNA targets. Signaling pathways and cellular processes were predicted using MienturNet (http://userver.bio.uniroma1.it/apps/mienturnet/) which infers evidence (computational or experimental) of microRNAs regulation on target genes based on a statistical analysis for over-representation of microRNA-target interactions

Statistical analysis

Experiments were performed three times by triplicate and results were represented as mean ± S.D. One-way analysis of variance (ANOVA) followed by Tukey's test were used to compare the differences between means. A $p < 0.05$ was considered as statistically significant.

Results

Identification of microRNAs involved in metastasis and vasculogenic mimicry

To initiate the study of microRNAs potentially involved in VM and metastasis, we first searched for differentially expressed microRNAs in a cohort of metastatic breast cancer patients from TGCA database (NCBI/GEO GSE68373) consisting of 64 tumor samples from 32 patients with metastasis and 32 with no distant metastasis. Data showed that 26 microRNAs showed significant variations in expression levels between metastatic and no-metastatic tumors (Fig. 1A). Prediction of gene targets of these microRNAs using TargetScan Human 8.0 identified around 40 gene targets involved in cancer cell migration, invasion and metastasis including ZEB1, ZEB2, serpine, MMP2, MMP14, ROCK1, NOTCH, STAT1, TGFB1, RAS, PI3K, AKT1, among others (Table 1). To define the importance of microRNAs during the metastasis of breast tumors, we constructed a microRNAs/mRNAs coregulation network for metastasis-associated microRNAs using miRNet tool (https://www.mirnet.ca/). Results showed that several important metastamiRs identified here may have a key role in the regulation of diverse gene targets involved in metastasis. For instance, we found that miR-942 was significantly downregulated in metastatic patients, and it was predicted to play roles in disseminated disease [17], as it may predicted to coordinately regulate master regulators of cell migration, invasion, epithelial-mesenchymal transition (EMT) and angiogenesis such as AKT, ZEB1, TGGB, EGFR, MMP2 and VGFA (Fig. 1B).

Likewise, a microRNAs/mRNAs coregulation network for microRNAs associated with VM was constructed. Data indicate that a set of specific microRNAs may regulate many gene targets involved in both VM and metastasis. For example, we found that miR-23c which was downregulated in metastatic patients, seems to regulate MMP2, MMP9, WNT11, and VGFA, which are involved in VM and angiogenesis (Fig. 2A). Analyses of the signaling pathways and cellular processes using MienturNet software which infers evidence, computational or experimental, of microRNAs regulation on targets based on a statistical analysis for over-representation of microRNA-target interactions, showed that microRNAs modulated in metastatic breast tumors may regulates oncogenic NOTCH, PI3K/AKT, MET, TGFB/SMADs, and FGFR3 signaling (Fig. 2B).

Vasculogenic mimicry in metastatic breast cancer

To establish a role of microRNAs in VM, we first analyzed breast tumors to obtain evidence of channel-likes structures formation in a small cohort of triple negative-breast cancer patients (Table 2). The 3D patterned matrix was identified using light microscopy of tumor sections stained with CD31 endothelial marker and periodic acid-Schiff (PAS). The channels-like structures were examined by immunohistochemistry assays using the CD31/PAS double staining on tissue sections of metastatic and non-metastatic tumors breast cancer patients. CD31 protein is an endothelial cell marker for classical blood vessels, whereas PAS staining is specific for glycoproteins enriched in extracellular matrix surrounding both blood vessels and channels-like structures formed by tumor cells.

In this way, channels-like structures (CD31-/PAS+) representative of VM could be distinguished from blood vessels which showed the CD31+/PAS+ immunophenotype in histologic samples. Data analysis showed a positive labeling for CD31 protein in endothelial cells in addition to intense pink color due to the PAS reaction in the walls that surround endothelial cells and, in channels formed by tumor cells in no-metastatic tumor samples (Fig. 3A, left panel). However, no CD31 signal was found in several PAS+ tissues indicating that channels observed correspond to the presence of VM in no-metastatic tissues (Fig. 3A, right panel). Likewise, data showed significant differences in the CD31/PAS staining intensities in tissues of metastatic patients (Fig. 3B). Remarkably, we observed an increase in CD31-/PAS+ stain of diverse channeled

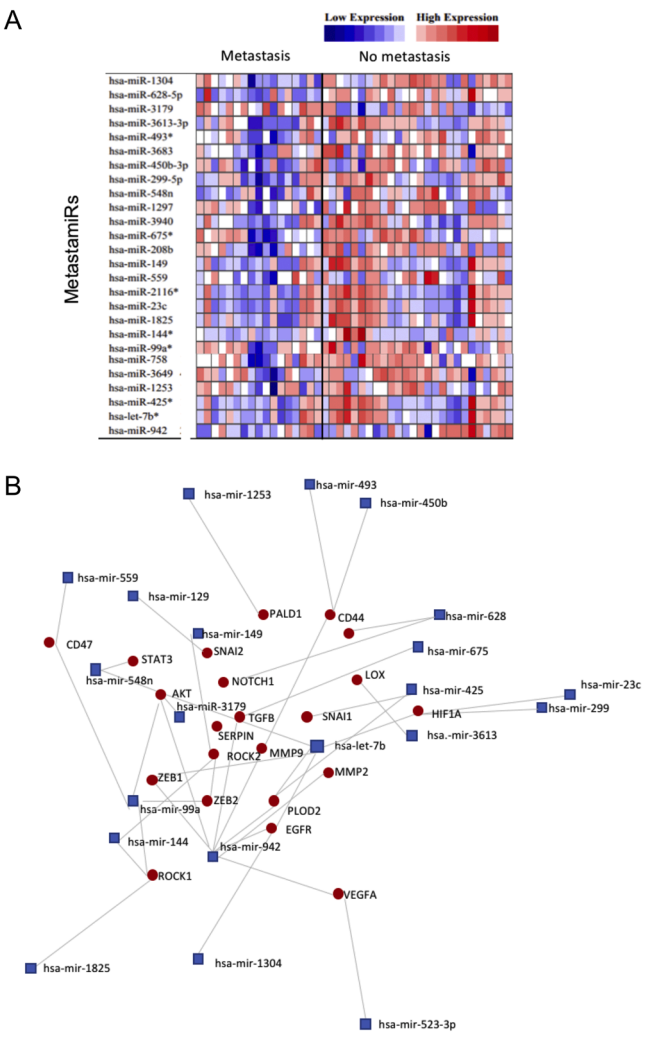

Fig. 1. MicroRNAs/mRNAs involved in metastasis. (A) Heath map showing the expression levels of microRNAs modulated in metastatic and no-metastatic breast cancer patients from TCGA database. (B) Coregulation network of microRNAs/mRNAs involved in metastasis constructed with miRNet on-line tool (https://www.mirnet.ca/).

Table 1
MicroRNAs involved in metastasis and vasculogenic mimicry.

MicroRNA	Expression in cancer	Potential targets involved in metastasis	Potential targets involved in VM
miR-9-3p	Downregulated	SNAI2, TWIST1, MMP16, VEGFC, AKT3, SOX9, GFB2, STAT3, SMAD2, JAK3, TGA2, E-CAD	TWIST1, HIF1A, MP16, VEGFC, AKT3, STAT3, SMAD2, EGR3 WNT, LMNB1, MAPK1
miR-126-3p	Downregulated	ITGA6, PIK3R1/2	ITGA6, AKT2
miR-29b-3p	Downregulated	VEGFA, MMP2, LOX, AKT3, TGFB2, GSK3B, STAT3, ITGA6, NOTCH2	VEGFA, MMP2, AKT3, NOTCH2, WNT, LAMA2
miR-145	Downregulated	ZEB2, LOX, AKT3, SOX9, TGFB2, SMAD2/3, ITGB8	AKT3, SMAD2/3, WNT
miR-125	Downregulated	AKT3, STAT3, SMAD2, ITGA	AKT3, SMAD2, ITGA, EPHA7
miR-148a-3p	Downregulated	MMP15, SOX5, TGFB2, SMAD2, ITGA9, ITGB8, EGFR	SMAD2, ITGA9, ITGB8, EPHA8, EGR3, WNT1, LAMA4
miR-526b	Downregulated	ZEB2, HIF3A, MMP1/2, LOX, TWIST1, AKT3, SOX, TGFR1/2/3, SMAD3, JAK1/2, ITGA4, ITGB8, EGFR, VECAM1, NOTCH2	MMP2, TWIST1, AKT3, SMAD3, ITGA4 ITGB8, NOTCH2, PHA5/7, WNT1/2/5, TFP
miR-142-3p	Downregulated	ZEB1/2, SOX5/11, TGFB2, ITGA8, ITGB8	ITGA8, ITGB8
miR-31	Downregulated	MMP16, SOX11	SRC, WNT
miR-200b-3p	Downregulated	SNAI2, ZEB1/2, VEGFA, MMP16, LOX, AKT2, SOX1/2, SMAD2, NOTCH1	VEGFA, AKT2, SMAD2, NOTCH1

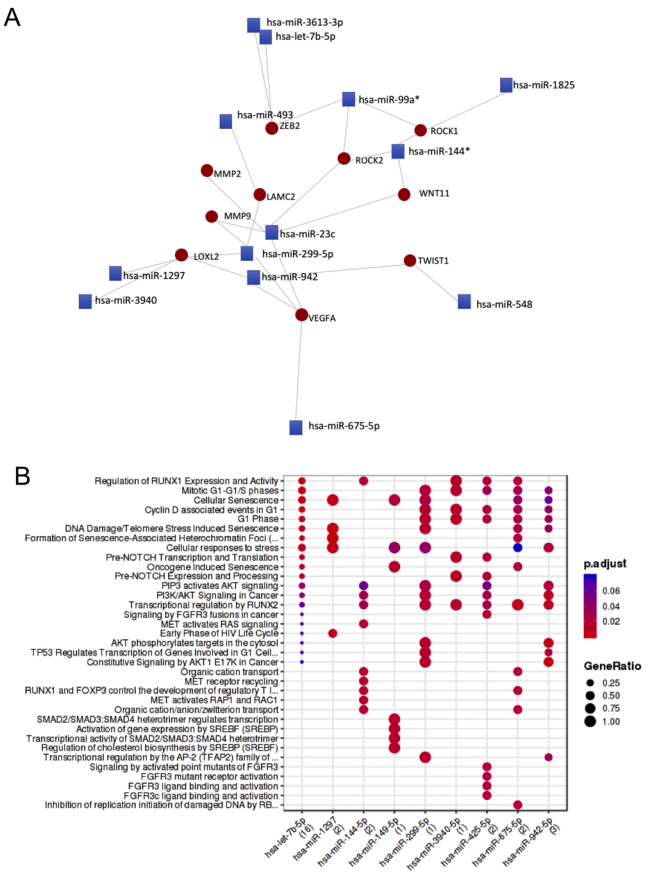

Fig. 2. MicroRNAs/mRNAs coregulation network associated with vasculogenic mimicry. (A) Coregulation network of miRNA/mRNA involved in VM constructed with miRNet on-line tool (https://www.mirnet.ca/). (B) Functional enrichment analysis of gene targets of mRNAs repressed in metastatic breast tumors using MienturNet (http://userver.bio.uniroma1.it/apps/mienturnet/).

structures in tissues from metastatic patients. Also, multiple erythrocytes within the lumen of channels-like structures were detected indicating that they could be functional transporting red blood cells, as previously described [6]. Quantification of the number of blood vessels showed no significant differences in vascularization between metastatic and no-metastatic tumors (Fig. 3C-E). In contrast, a significant increase in the number of channels-like structures in primary tumors from metastatic patients was found, suggesting that the metastasis event could be related with increased VM (Fig. 3C-E).

Expression of microRNAs associated to vasculogenic mimicry and metastasis in breast cancer patients

To determine the functions of microRNAs in VM in metastatic breast cancer, we analyzed the expression levels of the ten microRNAs (Table 1) using stem-loop qRT-PCR assays. The differential expression of microRNAs in non-metastatic compared to the metastatic group is shown in Fig. 4. Results showed that miR-142-3p, miR-31, miR-148a-3p, miR-145, miR-200b-3p, and miR-526b, showed a significant downregulation in metastatic group of tumors. In contrast, miR-9-3p and miR-125b-3p were upregulated in metastatic tumors, whereas no significant changes of miR-29b-3p and miR-126-3p levels between both groups were found (Fig. 4). A comparative analysis of the expression levels of the set of microRNAs with data reported in TCGA databases showed that only miR-142-3p and miR-miR-31 showed a similar regulation between metastatic and non-metastatic tumors, indicating the inherent heterogeneity in gene expression levels between tumors in breast cancer patients from different geographic locations. Also, we analyzed the mRNA expression of HIF-1 alpha a known driver of metastasis and microRNAs regulator in the same set of biopsies, and in a large cohort (n=1075) of breast cancer patients from UALCAN database. Data indicated no significant differences in HIF-1 alpha expression in metastatic and no metastatic groups positive for VM. In contrast, a slight but significant difference in mRNA expression was found between no metastatic and metastatic patients with different lymph nodes status in the large cohort of breast cancer patients (Supplementary figure S1 and S2).

MicroRNA-145 inhibits vasculogenic mimicry in breast cancer cells

We were wondering downregulated if miR-145 it could be involved in the formation of hypoxia-induced channels-like structures in triple negative breast cancer cells. Therefore, we evaluated the role of miR-145 in the formation of channels using a Geltrex based model that recapitulates the early stages of VM *in vitro,* as described in materials and methods. First, the expression of miR-145 was restored using transfection of RNA mimics, and subsequently cells were subjected to hypoxia for 48 h and seeded over Geltrex. The formation of VM structures was evaluated during 0-9 h course of time. Results showed that in both non-transfected and negative control-transfected MDA-MB-231 and Hs-578t breast cancer cells, channels-like structures were formed after the first 3 h after being seeded on ECM-rich matrix. In contrast, we observed that restoration of miR-145 expression levels resulted in a significant inhibition of formation of channels-like structures in both cell lines (Fig. 5 and 6). Quantification of branch points and channels-like showed that these structures increased during time in control conditions, whereas in miR-145-expressing cells they were significantly abolished in both breast cancer cell lines (Fig. 5 and 6).

Table 2
Clinical and pathological features of breast cancer patients.

Characteristics	Patients
Histological grade	
1	0 (0%)
2	2 (20%)
3	8 (80%)
Breast cancer staging	
IIA-IIB	1 (10%)
IIIA-IIIB	5 (50%)
IIIC-IV	4 (40%)
Receptor Status	0
Estrogen receptor	0
Progesterone receptor	0
HER2 receptor	
Molecular subtype	
Luminal A/B	0
Triple negative	10 (100%)
TNM staging	
T1	1 (10%)
T2	2 (20%)
T3	3 (30%)
T4	2 (20%)
N0	1 (10%)
N1	3 (30%)
N2	4 (40%)
N3	1 (10%)
M0	6 (60%)
M1	4 (40%)
Metastasis status	
No metastasis	6 (60%)
Metastasis	4 (40%)

Low expression of miR-145, miR-148a and miR-31 correlate with low overall survival

Then, we were wondering if changes in gene expression of microRNAs have clinical implications in breast cancer. Thus, we performed a overall survival analysis using Start miRpower Plotter for breast cancer as implemented in [18], which use genomic data and overall survival clinical information from a cohort of patients (n=1262) which have a long follow-up of median 94 months. Kaplan-Meier analysis showed that low levels of miR-145, miR-148a, and miR-31 in tumors were associated with low overall survival of patients (Fig. 7).

Discussion

Vasculogenic mimicry was reported for the first time by Maniotis in 1999 [4], Since then, the concept remains controversial as many cellular mechanisms and experimental approaches to study this phenomenon has not been solved, indicating the needs for new biomarkers of VM and criteria by which should be well defined using *in vitro* and *in vivo* models [19]. Metastasis remains as one of the more devastating cancer hallmarks, which account for most of cancer-related deaths. Notably, VM has been established a s a novel marker of poor prognosis in breast cancer patients, as it promotes resistance to therapy [20,21], and tumor progression [22]. Although the molecular mechanisms for VM development have been the objective of intense research, little is known about its regulation by ncRNAs. Indeed, very few studies indicate that microRNAs may regulate VM in breast cancer [23–26]. To fill this gap of knowledge and contribute on the understanding of the

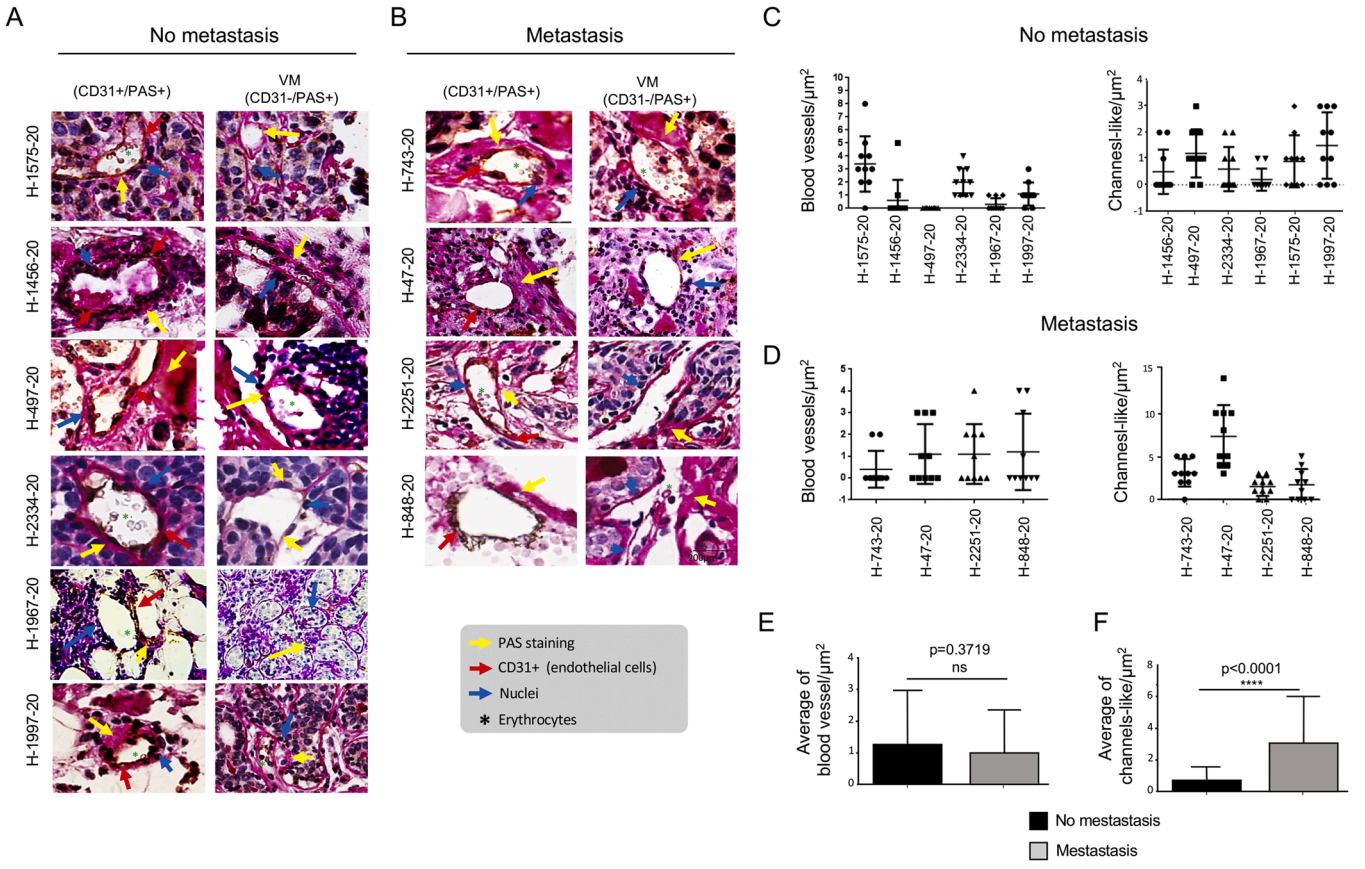

Fig. 3. Identification of channel-like structures in non-metastatic and metastatic triple negative breast cancer tissues. The (A) non-metastatic and (B) metastatic tissues showed blood vessels formed by endothelial cells (CD31+/PAS+), and channel-like structures (CD31-/PAS+) indicative of VM. Red arrows indicate positive CD31 labeling for endothelial cells, yellow arrows indicate positive PAS staining, blue arrows indicate tumor cells surrounding the vasculature, (*) indicate erythrocytes. Quantification of channels in primary tumors from (C) non-metastatic and (D) metastatic patients. (E-F) Average channels and blood vessels-like density in non-metastatic and metastatic patients.

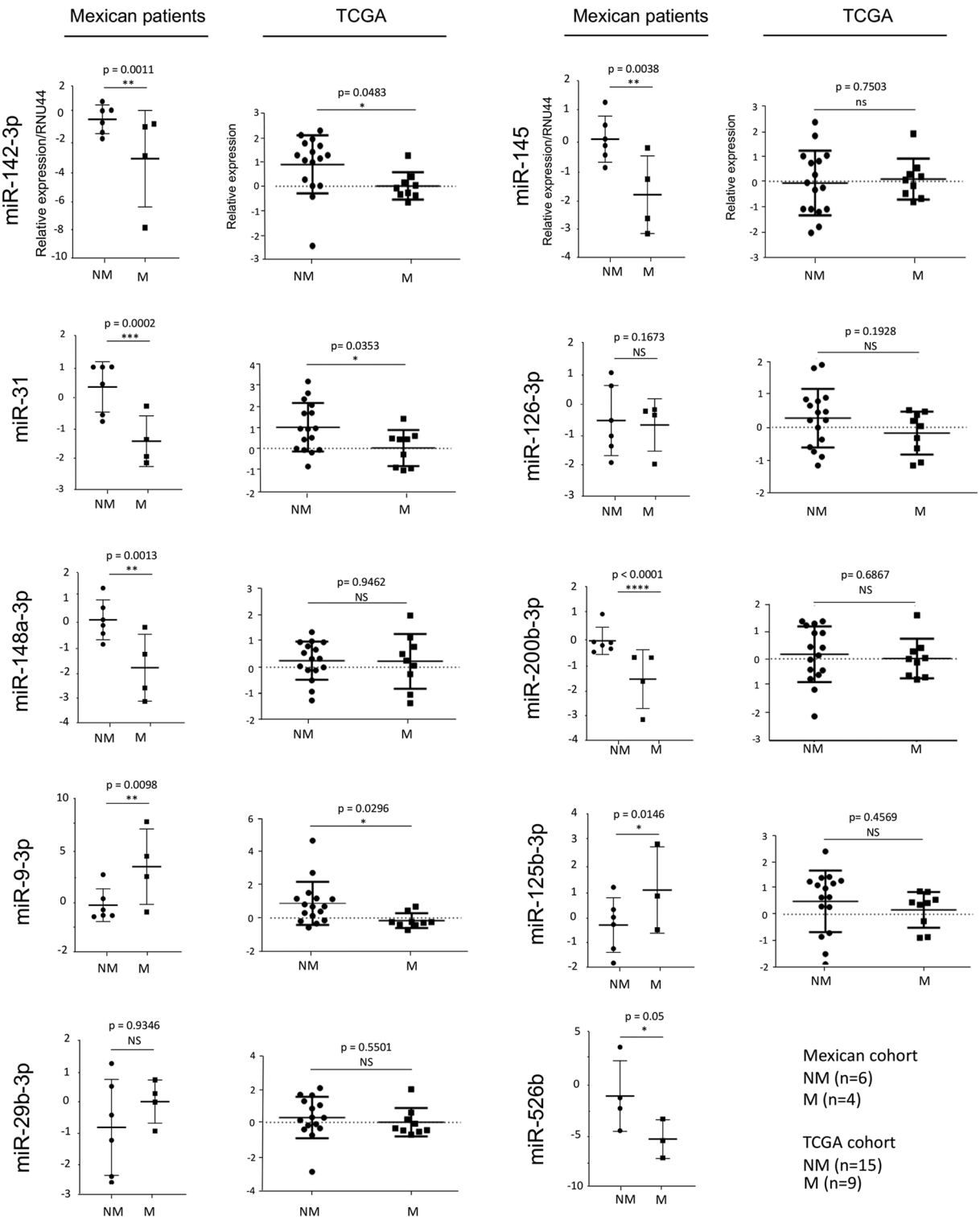

Fig. 4. Relative expression of microRNAs in non-metastatic and metastatic breast cancer patients. Quantitative RT-PCR assays showing the differential expression of ten microRNAs in tumors from non-metastatic patients compared with metastatic group. *p<0.05, **p<0.01, ****p<0.001.

posttranscriptional regulation of genes involved in VM and metastasis, here we analyzed the expression of ten microRNAs associated to both cancer hallmarks. Our data confirm that microRNAs are significantly modulated in metastatic tumors which display great abilities to form channel-like structures. Of clinical interest, we observed that low expression of miR-145, miR-148a and miR-31 correlated with low overall survival in breast cancer patients, which suggest that they could be markers of poor prognosis. We focused on the study of miR-145, a tumor suppressor gene in multiple types of malignancies such as bladder, breast, cervical, renal cancer, and gastrointestinal cancers [27], as it was severely suppressed in metastatic tumors exhibiting VM. Interestingly, VM has been associated with resistance to anti-cancer therapies, this may be due to the repression of miR-145, as a recent study demonstrate that it attenuates paclitaxel resistance and suppressed cancer progression by targeting SOX2 [28]. In addition, low levels of miR-145 were also associated with resistance to cisplatin by

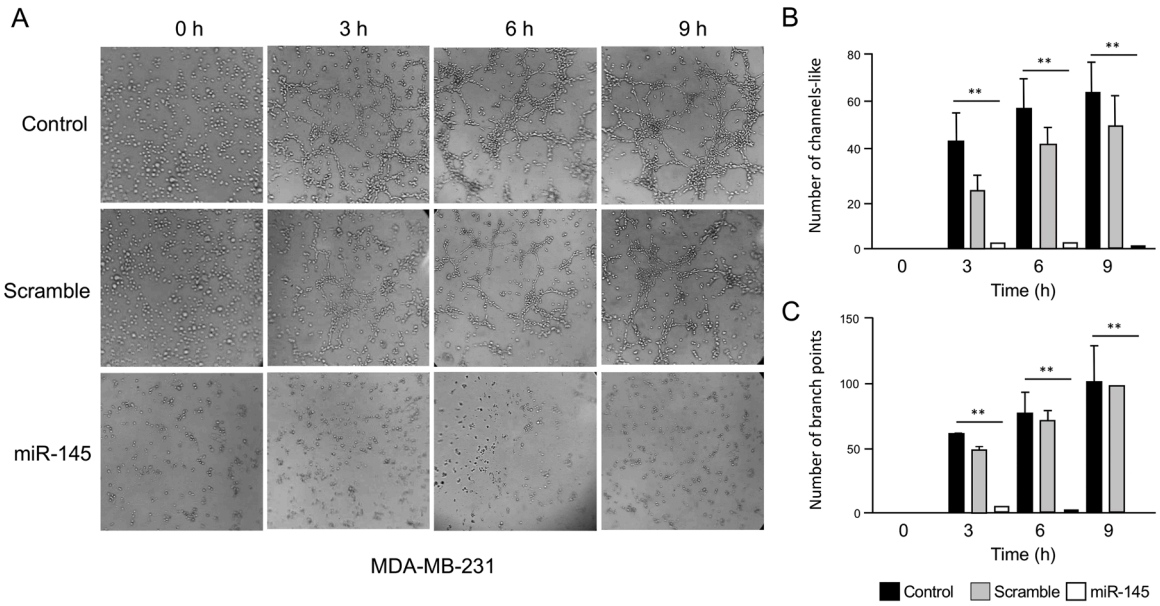

Fig. 5. MiR-145 inhibits vasculogenic mimicry in MDA-MB-231 cells. (**A**) Optical microscopy images of MDA-MB-231 cells treated with miR-145 precursor (Bottom panel), scrambled (middle panel), and non-transfected control (Upper panel). (**B,C**) Graphical representation of the number of branch points and channels-like structures from panel A. Experiments were performed three times by triplicate and data were expressed as mean ± S.D.
$^{**}p < 0.05$

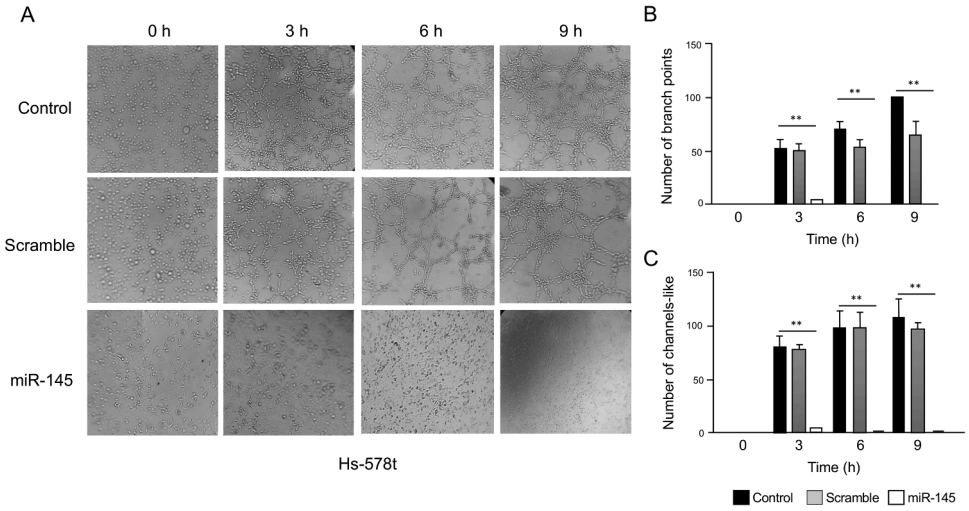

Fig. 6. MiR-145 inhibits vasculogenic mimicry in Hs-578t cells. (**A**) Optical microscopy images of Hs-578t cells treated with miR-145 precursor (Bottom panel), scrambled (middle panel), and non-transfected control (Upper panel). (**B, C**) Graphical representation of the number of branch points and channels-like structures from panel A. Experiments were performed three times by triplicate and data were expressed as mean ± S.D. $^{**}p<0.05$.

targeting TGFBR2 receptor and poor pathological complete response in breast cancer patients [29]. Moreover, miR-145 expression and functions have been associated with cell migration and invasion. Hu and coworkers reported that miR-145-5p inhibited the proliferation, migration, and invasion of BC cells via posttranscriptional repression of HMGB3 [30]. All these reports, and our own data suggested that miR-145 could be a good prognosis biomarker in breast cancer. In addition, previous studies in mice models reported about the in vivo role for miR-145 in tumor growth [31]. Also, an interesting study from Mallet and coworkers demonstrate that polyphenols from Blueberry fruit were capable to abolish the spheroids formation from tumoral primary cells in tumors removed from animals treated with polyphenols which was associated with a significant increase of miR-145 expression [32].

Transcription factor HIF-1 alpha is a well-known driver of tumor development and progression influencing several cancer hallmarks including angiogenesis, vasculogenic mimicry and metastasis [33]. Intriguingly, we found no substantial changes in mRNA expression of HIF-1 alpha between metastatic and no metastatic tumors positive to vasculogenic mimicry (Supplementary Data S1). However, the results it could be no convincing as we used a small number of samples, thus we decided to analyze the HIF1-alpha expression in clinical tumors using UALCAN datasets from a large cohort of metastatic and no-metastatic breast cancer (n=1075). Data showed slight but significant differences in HIF-1 alpha mRNA expression between no metastatic (N0) and metastatic patients with different lymph nodes status (N1,N2,N3), suggesting that HIF-1 alpha expression it could be regulated by additional molecular mechanisms, maybe at posttranslational level by stabilization of HIF-1 alpha protein resulting from post-translational modifications such as hydroxylation, ubiquitination, acetylation, and phosphorylation

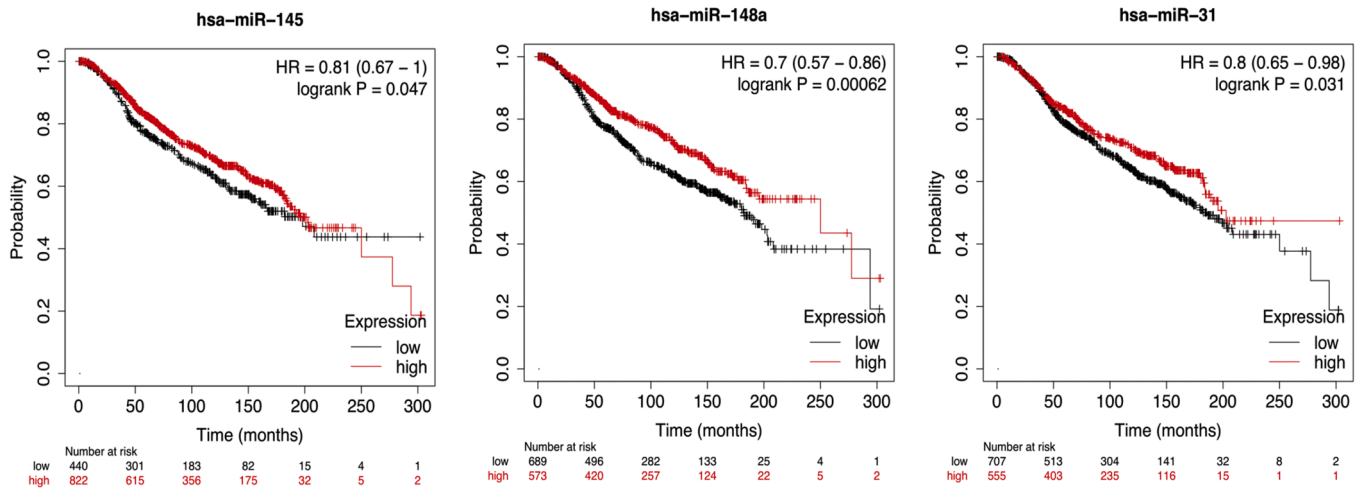

Fig. 7. Kaplan-Meier curves for overall survival according to the expression of miR-145, miR-148a and miR-31. Overall survival analysis was performed using Start miRpower plotter for breast cancer tool that use the genome-wide for mRNA expression data and overall survival clinical information of cancer patients. Kaplan-Meier survival plots compared the two patient cohorts, and the hazard ratio with 95% confidence intervals and logrank P value were calculated as described [18].

in response to hypoxia, as it has been previously reported [33].

The main disadvantage of our study is the small number of patients analyzed here, but we considered important our preliminary findings in tumor tissues, as no previous relationships between metastasis/no-metastasis events and vasculogenic mimicry have been reported in breast cancer. Therefore, we will consider this first study as pilot, with more specimens to validate these results in a future project. The main advantages of the present report are related to the clinical implications of our findings which pointed out to the urgence of novel and effective treatments for metastatic disease positive to VM in breast cancer patients, suggesting that VM evaluation in biopsies must be considered as a novel clinical marker of prognosis in metastatic disease. Moreover, as we reported here the in vitro targeting of miR-145 expression was able to abolish the VM in cancer cells, which is an important cellular mechanism for fueling tumor growth, suggesting that manipulation of microRNAs expression could represent an alternative therapy in breast cancer. Although the functional and clinical links between VM and metastasis remains still obscure, our data suggested that microRNAs regulation has an important role in the regulation of the vascular-like related processes associated with metastasis.

Conclusion

In conclusion, manipulation of miR-145 levels may represent a therapeutic approach in metastatic breast cancer patients that developed vasculogenic mimicry, while simultaneously creating new potential vulnerabilities.

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CRediT authorship contribution statement

Estefania Contreras-Sanzón: Investigation, Methodology, Formal analysis. **Ángeles Carlos-Reyes:** Investigation, Methodology, Formal analysis. **Mónica Sierra-Martínez:** Investigation, Data curation, Resources. **Gustavo Acosta-Altamirano:** Investigation, Data curation, Resources. **Cesar Luna-Rivero:** . **David Núñez-Corona:** Investigation, Methodology, Formal analysis. **Alejandra Paola García-Hernández:** Investigation, Formal analysis. **Eloisa Ibarra-Sierra:** Data curation, Resources. **Horacio Vidrio-Morgado:** Data curation, Resources. **María Elizbeth Alvarez-Sánchez:** Investigation, Formal analysis. **Laurence A. Marchat:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing. **César López-Camarillo:** Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2023.101680.

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