Melatonin restrains angiogenic factors in triple-negative breast cancer by targeting miR-152-3p: *In vivo* and *in vitro* studies

Jéssica H.M. Marques[c], André L. Mota[c], Jessica G. Oliveira[a,c], Jéssica Z. Lacerda[b,c], Júlia P. Stefani[c], Lívia C. Ferreira[c], Tialfi B. Castro[c], Andrés F. Aristizábal-Pachón[d], Debora A.P.C. Zuccari[a,b,c,*]

[a] *Graduate Program in Health Science, Faculdade de Medicina de Sao Jose do Rio Preto - FAMERP, Sao Jose do Rio Preto, SP, Brazil*
[b] *Graduate Program in Biosciences, Universidade Paulista-UNESP/IBILCE, Sao Jose do Rio Preto, SP, Brazil*
[c] *Laboratory of Molecular Research in Cancer – LIMC, Faculdade de Medicina de Sao Jose do Rio Preto - FAMERP, Sao Jose do Rio Preto, SP, Brazil*
[d] *Laboratory of Molecular Genetics and Bioinformatics – LGMB, Faculdade de Medicina da Universidade de Sao Paulo, Ribeirão Preto, SP, Brazil*

A B S T R A C T

Aims: Breast cancer represents the second most prevalent tumor-related cause of death among women. Although studies have already been published regarding the association between breast tumors and miRNAs, this field remains unclear. MicroRNAs (miRNAs) are defined as non-coding RNA molecules, and are known to be involved in cell pathways through the regulation of gene expression. Melatonin can regulate miRNAs and genes related with angiogenesis. This hormone is produced naturally by the pineal gland and presents several antitumor effects. The aim of this study was to understand the action of melatonin in the regulation of miRNA-152-3p *in vivo* and *in vitro*.
Main methods: In order to standardize the melatonin treatment in the MDA-MB-468 cells, we carried out the cell viability assay at different concentrations. PCR Array plates were used to identify the differentiated expression of miRNAs after the treatment with melatonin. The relative quantification of the target gene expression (IGF-IR, HIF-1α and VEGF) was performed by real-time PCR. For the tumor development, MDA-MB-468 cells were implanted in female BALB/c mice, and treated or not treated with melatonin. Moreover, the quantification of the target genes protein expression was performed by immunocytochemistry and immunohistochemistry.
Key findings: Relative quantification shows that the melatonin treatment increases the gene expression of miR-152-3p and the target genes, and decreased protein levels of the genes both *in vitro* and *in vivo*.
Significance: Our results confirm the action of melatonin on the miR-152-3p regulation known to be involved in the progression of breast cancer.

1. Introduction

Breast cancer (BC) represents the second most prevalent type of tumor with the highest mortality rate in the world among women. Annually, more than one million women are diagnosed with breast cancer and > 400,000 die from this disease [1]. The evolution of this neoplasm occurs when the cells break off and spread to other regions of the body [2]. In this case, there is a need for recruitment of new blood vessels by angiogenesis [3,4]. Tumor angiogenesis is a very complex process and can be regulated by several mechanisms involving different cell types of the tumor microenvironment that release pro-angiogenic factors such as vascular endothelial growth factor (VEGF) [5].

During the cancer progression, when the tumor exceeds 1–2 mm in diameter, hypoxia regions are formed [6]. Moreover, the hypoxia increases the expression of pro-angiogenic factors such as pVHL and HIF-1α, or even control epigenetic mechanisms involving microRNAs (miRNAs) [7]. Recent studies demonstrate that the increase of IGF-IR (Insulin-like growth factor 1 receptor) in angiogenesis might be related to other genes, such as HIF-1α (Hypoxia-Inducible Factor) and VEGF leading to angiogenesis [8,9].

Several studies suggest that melatonin is capable of modifying the expression of innumerable genes related to breast cancer [10–13], including studies in metastatic processes, cell-cell and cell-matrix interaction, and the epithelial-mesenchymal transition [14]. Melatonin, a

hormone naturally produced and secreted in the pineal gland and whose synthesis is blocked in the presence of light [15], has been shown to be important as a new therapy against breast cancer. In addition, this hormone has important functions including antiangiogenic effects [16] as confirmed by our research group *in vitro* and *in vivo* [17].

miRNAs are endogenous small molecules of non-coding RNA, composed of 19–24 nucleotides that act in the cellular pathways through the regulation of gene expression in post-transcriptional level. These molecules can induce gene silencing through specific pairing with target messenger RNA (mRNA) and culminate in its degradation or transcriptional repression. It is already known that one gene can be repressed by a wide variety of miRNAs, while simultaneously one miRNA can regulate several target genes [18–20].

In breast tumors, miRNAs can act as tumor suppressors or oncogenes, regulating several genes that lead to cell proliferation, apoptosis, genomic instability, metastasis, angiogenesis and tumor growth [21,22]. Among miRNAs, the miR-152 demands special interest, because its decrease has been associated with the process of cell proliferation, invasion and angiogenesis in different neoplasms such as breast neoplasm [9]. In breast cancer, miR-152-3p levels are relatively decreased compared to normal mammary tissues. This miRNA can act on the pathway of angiogenesis, targeting IGF-IR and IRS1, which leads to the inhibition of some signalling pathways in the cell, culminating in the inhibition of HIF-1α and VEGF, factors that promote the synthesis of new blood vessels [9,23].

There are no studies evaluating the effect of melatonin on the expression of miRNAs in triple-negative breast cancer (TNBC). In this manuscript, we investigated how melatonin impairs angiogenesis, increasing microRNA-152-3p in triple-negative breast cancer cell line. Our findings suggest a modulating role of melatonin in the tumor suppressor miR-152-3p and in genes related to angiogenesis.

2. Materials and methods

2.1. Cell culture

TNBC MDA-MB-468 cell line was cultured in 5% CO_2 at 37 °C in Dulbecco's Modified Eagle's Medium-High Glucose (DMEM®) medium, supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin and Streptomycin (LGC Biotecnologia, SP, BR).

2.2. Cell viability assay (MTT)

MDA-MB-468 cells at a concentration of 5×10^4 were placed in individual wells of a 96-well plate and incubated for 24 h in DMEM with 2% FBS. The treatments were performed using four different concentrations of melatonin (0.001 mM, 0.01 mM, 0.1 mM and 1 mM) (Sigma-Aldrich, St. Louis, MO, USA). Control group received only the vehicle (1: 1–100% ethanol and PBS). After 48 h of treatment, 10 μL of MTT solution (Vibrant Mtt Cell Proliferation Assay Kit-Invitrogen®) was added to each sample, followed by incubation for 1 h. Subsequently, for solubilization of the crystals formed from the metabolism of MTT, the cells were incubated with 100 μL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) for 10 min. The absorbance was measured at 570 nm wavelength using the FLUOstar Omega Microplate Reader® plate reader.

2.3. Real time quantitative PCR (RQ-qPCR)

RNA samples were extracted from the cell line using the miRNeasy Mini Kit® (QIAGEN, Hilden, GER) following the manufacturer's guidelines. The QIAzol reagent® and the extraction kit were used to preserve the miRNAs that could be dissipated in a conventional total RNA extraction. The cDNA was obtained through miScript II RT Kit (QIAGEN, Hilden, GER). The qPCR was performed using the RT^2 Profiler™ PCR Array Human Breast Cancer; this array is composed of 84 mature miRNAs related with breast cancer and controls. StepOnePlus real-time PCR (Applied Biosystems, Foster City, CA, USA) was used and fluorescence data were collected during the extension step. We determined specific gene expression for miRNA and its targets. The cDNA (single strand - complementary DNA) was obtained using the TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) for miRNAs, and the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) for genes, according to the manufacturer's specifications. The quantitative real-time polymerase chain reaction (qPCR) was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Reactions for miR-152-3p and its target genes (IGF-IR, HIF-1α and VEGF) expression analysis were performed in triplicate using TaqMan™ Universal PCR Master Mix, TaqMan™ RNA Assay (Applied Biosystems, Foster City, CA, USA) and 10 ng of cDNA. To normalize miRNA and mRNA expression, we used housekeeping genes U6 and β-actin, respectively. The relative expression values of the miRNAs of interest were determined by the quantification method in relation to the mean of normalizing genes used as endogenous control ($2^{-\Delta\Delta Ct}$).

2.4. Transient modification of cells

In order to perform the transient modification, the MirVana™ miRNA Mimic 152-3p (Ambion®) was used; this reagent is made up of small molecules of double-stranded RNAs that mimic an endogenous miRNA and allows functional analyses due to its overexpression. Additionally, a negative control containing a nucleotide sequence without homology to any gene (scramble) described in mammals was used. The MDA-MB-468 cells were seeded in 6-well plates and transfected using HiPerFect Transfection Reagent (QIAGEN, Hilden, GER).

2.5. Immunocytochemistry

The protein expression of the target genes IGF-IR, HIF-1α and VEGF was performed by immunocytochemical assay (ICC). The cell line was transferred to a slide with coupled silicone, where the culture medium was added, treated and transfected for 24 h. After the medium was removed, the slide was incubated overnight with 250 μL of 4% paraformaldehyde. The primary antibodies were used (Table 1) and the slides were incubated at 4 °C overnight. The Complement and HRP Conjugate (REVEAL-Biotin-Free Polyvalent DAB-Spring Bioscience, Pleasanton, CA) were applied, followed by the chromogenic substrate (DAB) and Harris Hematoxylin. The assembly of the slides was performed in 50% glycerol and sealed. All immunoreactions were accompanied by a positive control for the antibody tested and a negative control (no primary antibody). The slides were observed on the 40× objective (Nikon Eclipse E200®) microscope and analyzed by optical densitometry. For each sample, three different fields were

Table 1

Primary antibodies used in immunocytochemistry and immunohistochemistry techniques and their respective applications.

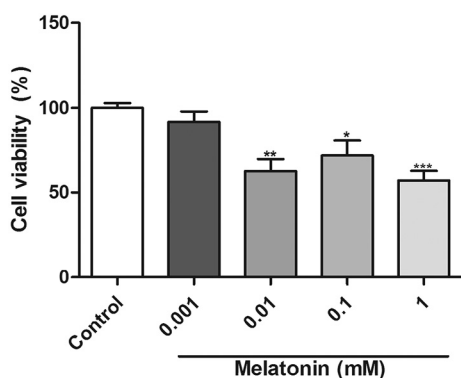| Antibody	Company	Clone	Dilution ICC	Dilution IHC	Marking
IGF-IR	Sigma-Aldrich, St. Louis, MO, USA	C-terminal	1:50	1:400	Nuclear and cytoplasmatic
VEGF	Santa Cruz Biotechnology, Dallas, TX, USA	A-20	1:25	1:50	Nuclear and cytoplasmatic
HIF-1α	Santa Cruz Biotechnology, Dallas, TX, USA	H1alpha67	1:25	1:40	Cytoplasmatic

Fig. 1. Melatonin modifies cell viability of MDA-MB-468 cells. The cell viability was performed by MTT assay. The MDA-MB-468 cells were treated for 48 h with different concentrations of melatonin (0.001 mM, 0.01 mM, 0.1 mM and 1 mM). The ideal concentration of melatonin for treatment was 1 mM. The results express the distribution model according to the three experiments performed. The data reveal the mean ± the Standard Error of Mean (SEM) (*p < 0.05, **p < 0.01 and ***p < 0.001 treatment *versus* control) (ANOVA one-way and post-hoc Bonferroni).

photographed only in the immunoreactive areas, and IMAGE J® software was used to quantify the immunostained intensity.

2.6. Animals and tumor implantation model

Female BALB/c nude mice (body weight of 20–25 g) were used. The animals were kept in pathogen free conditions in a temperature-controlled environment (21 to 25 °C), exposed to light for 12 h and 12 h in the dark, and given food and water *ad libitum*. Mice were acquired from Faculdade de Medicina da Universidade de Sao Paulo (FMUSP) and the experiment was carried out at Faculdade de Medicina de Sao Jose do Rio Preto (FAMERP). MDA-MB-468 cells were trypsinized, centrifuged and suspended in 100 μL of serum free DMEM at the concentration of 5×10^6 cells. For the development of the tumor, this volume was injected subcutaneously in the flank. The treatment was performed for 21 days (during week days) using 40 mg of melatonin/kg animal weight. Melatonin was diluted 1:1 PBS/100% ethanol and applied intraperitoneally just before the light was switched off, at the end of the light phase. After the treatment period, the animals were euthanized with pentobarbital overdose (100 mg/kg). Tumor tissue was removed, and one part was used for real-time PCR, while another part was fixed in 10% formalin for histological and immunohistochemical analysis. The study was carried out following the national and international standards for ethics in animal experimentation. The project was approved by the Ethics Committee on the Use of Animals of the Faculdade de Medicina de Sao Jose do Rio Preto (02/2015).

2.7. Immunohistochemistry

The protein expression of the target genes IGF-IR, VEGF and HIF-1α was performed by immunohistochemistry assay. The tumor tissue was paraffin embedded, cut into a 3 μm slice and placed on a silanized slide. The deparaffinization of the xylol sections was carried out followed by hydration with decreasing ethanol. Endogenous peroxidase blockade was performed with 10 V oxygenated water for 30 min and antigenic recovery in a steam pan for 30 min with buffer, as indicated by the manufacturer. The material was incubated with the primary antibodies and the corresponding concentrations (Table 1) in a darkroom for 18 h at 4 °C. After the incubation period, the slides were washed with saline (PBS), incubated with secondary, tertiary antibody, and developed with DAB chromogen according to instructions from REVEAL-Biotin-Free Polyvalent DAB-Spring (Bioscience, Pleasanton, CA). The counterstaining was performed with Harris Hematoxylin for 40 s, and assembly

of the slides in Erv-mount resin (Erviegas, Sao Paulo, SP, BR). The immunoreactions were accompanied by a positive control and a negative control. The slides were observed under a 40× objective of the Nikon Eclipse E200® microscope and analyzed by optical densitometry. For each sample, three different fields were photographed in the immunoreactive areas only, and IMAGE J® software was used to quantify the immunostained intensity.

2.8. Statistical analysis

The results were initially submitted to descriptive analysis for determination of normality. For samples with normal distribution, Student's *t*-test (two samples) or Analysis of Variance (ANOVA), followed by Bonferroni test (more than two samples) were used. Data were presented as mean ± Standard Error of Mean (SEM). Values of p < 0.05 were considered significant and all analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Melatonin affects cell viability

Cell viability was evaluated for different concentrations of melatonin to determine cytotoxic effects by MTT assay. Analysis shows that melatonin caused a significant decrease (p < 0.05) of cell survival compared to control group in concentration equal or higher than 0.01 mM. We chose the 1 mM concentration for the further experiments because it was more significant (p < 0.001) (Fig. 1).

3.2. Melatonin regulates miRNAs

We evaluated 84 mature miRNAs expression level in MDA-MB-468 cells by PCR-array. Table 2 shows the fold change (FC) value for 13 different miRNAs regulated for melatonin treatment (p < 0.05). Six were up-regulated including hsa-let-7c-5p, hsa-miR-152-3p, hsa-miR-182-5p, hsa-miR-202-3p, has-miR-214-3p, hsa-miR-29b-3p; seven were down-regulated including hsa-miR-107, hsa-miR-10a-5p, hsa-miR-145-5p, hsa-miR-15b-5p, hsa-miR-20a-5p, hsa-miR-429 and hsa-miR-7-5p. Among the miRNAs differentially regulated by melatonin, a significant increase was observed in miR-152-3p level expression (FC and p value). This miRNA was chosen for the next steps because it has scientific relevance in breast cancer and angiogenesis process [24].

3.3. Melatonin increases miR-152-3p and target genes expression

We validated by RT-qPCR the higher expression of miR-152-3p in

Table 2
Expressed miRNAs after melatonin treatment.

	Fold change	p value
Up-regulated miRNAs		
hsa-let-7c-5p	1,18	0,023
hsa-miR-152-3p	1,80	0,019
hsa-miR-182-5p	1,25	0,013
hsa-miR-202-3p	1,24	0,024
hsa-miR-214-3p	1,64	0,034
hsa-miR-29b-3p	1,11	0,041
Down-regulated miRNAs		
hsa-miR-107	0,83	0,007
hsa-miR-10a-5p	0,58	0,047
hsa-miR-145-5p	0,91	0,039
hsa-miR-15b-5p	0,94	0,011
hsa-miR-20a-5p	0,75	0,041
hsa-miR-429	0,62	0,003
hsa-miR-7-5p	0,50	0,026

In vitro - MDA-MB-468 cells

(A)

In vivo - Animals

(B)

Control
Melatonin
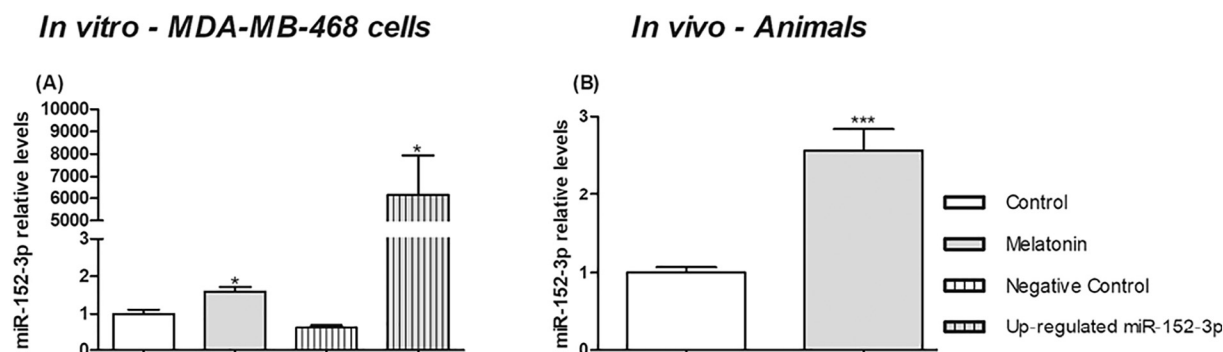Negative Control
Up-regulated miR-152-3p

Fig. 2. Melatonin increases the relative expression of miR-152-3p *in vitro* and *in vivo*. MDA-MB-468 cells were transfected with Mimic miR-152-3p and subjected to treatment with melatonin (1 mM) for 24 h. Furthermore, the cells without transfection were implanted in BALB/c nude mice and treated with melatonin (1 mM). Ten animals were used, five treated with melatonin and five treated with vehicle of melatonin. The results showed that melatonin increases expression of miR-152-3p (A, B). The data were determined on a log2 scale, since fold change varies with the mean of the Ct $\pm$ SEM of the triplicates of the groups after analysis ($*p < 0.05$ and $***p < 0.0001$ treatment *versus* control).

Control
Melatonin
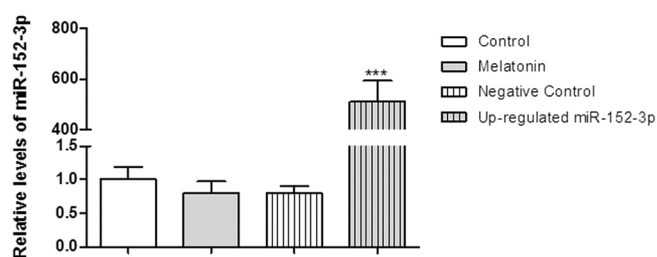Negative Control
Up-regulated miR-152-3p

Fig. 3. Melatonin does not increase the relative expression of miR-152-3p in MDA-MB-231. MDA-MB-231 cells were transfected with Mimic miR-152-3p and subjected to treatment with melatonin (1 mM) for 24 h. The results showed that melatonin does not increase expression of miR-152-3p. The data were determined on a log2 scale, since fold change varies with the mean of the Ct $\pm$ SEM of the triplicates of the groups after analysis ($***p < 0.0001$ treatment *versus* control).

MDA-MB-468 cells in different conditions, and also observed that the expression is increased in cells treated with melatonin (Fig. 2A) ($p < 0.05$). To identify the effect of melatonin in breast tumor development, we ectopically expressed miR-152-3p in MDA-MB-468 cell line. We consistently achieved 80% transfection efficiency in this cell line and miRNA expression reached above 6000 (Fig. 2A) ($p < 0.05$) in comparison to control cells, without transfection or treatment. The action of melatonin was also verified in xenograft model after implantation of MDA-MB-468 cell line. Melatonin was able to increase the expression of miR-152-3p in tumor tissue (Fig. 2B) ($p < 0.001$) in comparison with tumor tissue without treatment. To compare the results found with the MDA-MB-468 cell line, miR-152-3p was also investigated in the MDA-MB-231 cells, a similar triple-negative breast cancer cell line. The reagent increases miRNA expression ($p < 0.0001$), but melatonin does not modulate the same (Fig. 3).

3.4. Melatonin and miR-152-3p decreases target genes protein expression

Evaluation of protein expression by immunocytochemistry showed that melatonin and up-regulated miR-152-3p decreased the protein levels of IGF-IR ($p < 0.001$ and $p < 0.0001$), HIF-1α ($p < 0.001$ and $p < 0.0001$) and VEGF ($p < 0.05$ and $p < 0.0001$) in MDA-MB-468 cells (Fig. 4A–C). Protein expression measured by immunohistochemistry revealed reduced levels of HIF-1α ($p < 0.001$) and VEGF ($p < 0.05$) (Fig. 4E, F) in melatonin treated groups *in vivo*. The slight reduction observed for IGF-IR (Fig. 4D) was not statistically significant, although it was possible to observe a more pronounced marking in the control group compared to the melatonin treatment.

4. Discussion

Melatonin appears to play a key role in protecting against breast cancer. This hormone interacts with several transcription factors and in nuclear binding sites, contributing to the reduction of cell proliferation [25]. Several studies attribute to melatonin the inhibition of angiogenesis by reducing the genes related to this process. Melatonin inhibits angiogenesis in co-cultures of human endothelial cells (HUVECs) and breast cancer (MCF-7) [26,27]. It has already been demonstrated by our group that melatonin regulates angiogenic proteins in the MDA-MB-231 cell line and in the co-culture with cancer-associated fibroblasts [28]. This hormone also regulates angiogenesis under hypoxia in MCF-7 and MDA-MB-231 cells [29] and decreases the expression of genes related to this process in MDA-MB-231 and MCF-7 cells in three-dimensional culture [30]. Thus, genes related to angiogenesis can be regulated by various mechanisms and molecules, including miRNAs [31] and melatonin [32].

There are no studies investigating the action of melatonin on miRNAs in MDA-MB-468 cells. Our results demonstrate the therapeutic potential of melatonin in the control of angiogenesis, in post-transcriptional gene regulation by increasing miR-152-3p expression in MDA-MB-468 cells, a triple-negative breast cancer cell line (TNBC), and in decreasing the miRNA target genes (IGF-1R, HIF-1α and VEGF) protein expression. We also identified the same results after tumoral implantation of these cells in BALB/c mice. Tumors treated with melatonin slightly decrease in growth relative to the vehicle-treated group (PBS/ethanol), data not shown. We have shown that this hormone acts by regulating miRNAs and genes related to angiogenesis in triple-negative breast cancer (TNBC).

We chose a triple-negative breast cancer cell line for this study, MDA-MB-468, because triple-negative tumors are characterized by limited treatment options, leading to poor prognosis and high host mortality [33]. This lineage has already been used in studies related to the epithelial-mesenchymal transition (EMT) [34]. In addition, some molecules were investigated in this cell line, such as (Boc) 2-creatine and metformin in the ATP/AMP ratio which decreased cell viability [35]; the leptin promoting EMT in breast cancer cells [36], MT3-037 (drug in study) and sunitinib which inhibited angiogenesis [37,38]. Through the MT1 and MT2 receptors, melatonin stimulates apoptosis, regulates survival signalling and tumor metabolism, and also inhibits angiogenesis and metastasis [39,40]. In addition, since melatonin is a derivative of the amino acid tryptophan and can easily cross biological membranes due to its amphipathic nature, it can still be transported by glucose transporters [39,41], performing its functions independent of its MT1 and MT2 receptors. The low expression of MT1 receptors in triple-negative tumors has already been identified [40], and Mao et al. showed mechanisms involved in the resistance of MDA-MB-231 cells to

In vitro – MDA-MB-468 cells
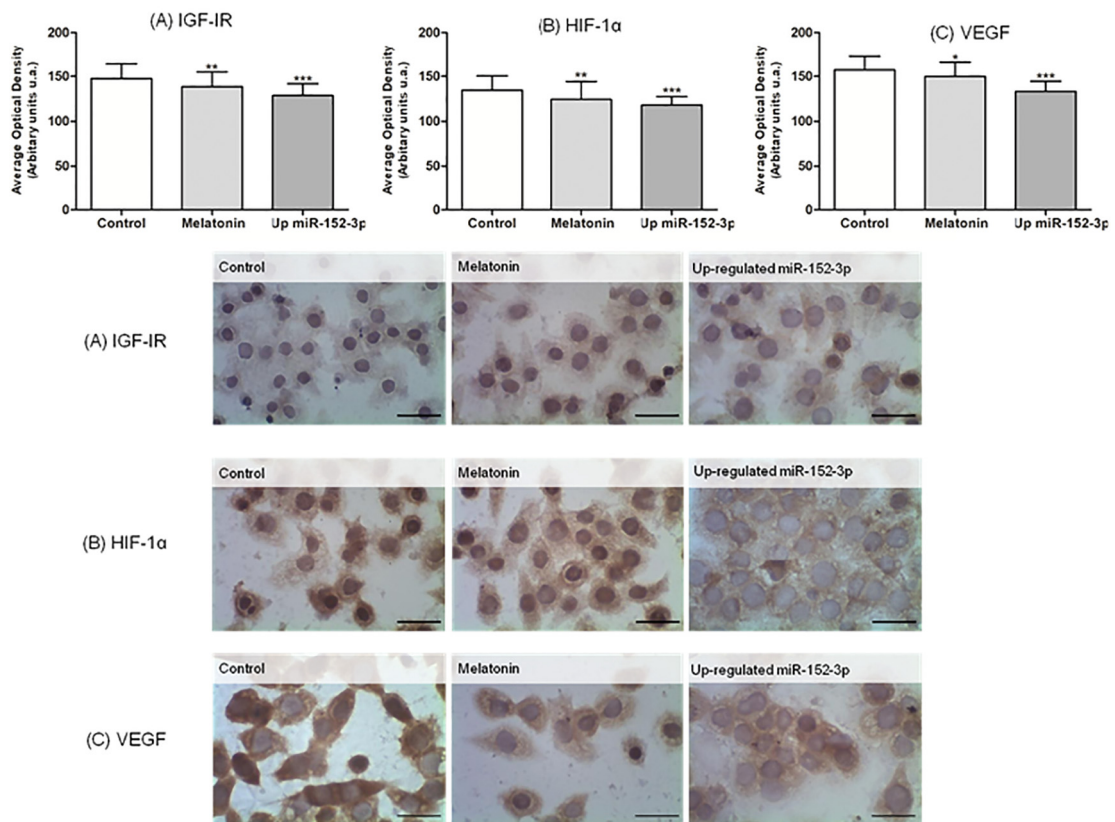
In vivo – animals
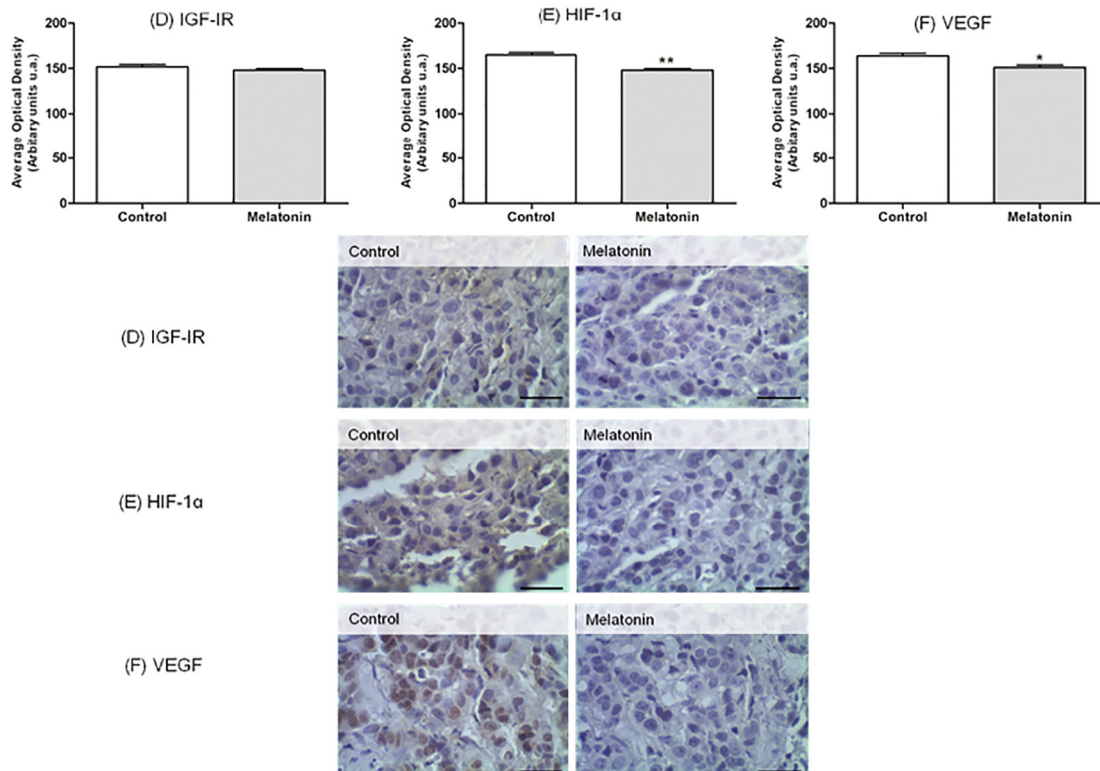
(caption on next page)

Fig. 4. Melatonin decreases protein expression of IGF-IR, HIF-1α and VEGF by ICC and IHC. MDA-MB-468 cells were transfected with Mimic miR-152-3p and subjected to treatment with melatonin (1 mM) for 24 h. The results showed that it decreases protein expression of IGF-IR, HIF-1α and VEGF *in vitro* and *in vivo* (A–F). The up-regulated miR-152-3p decreased the protein expression of the target genes *in vitro* (A–C). The values express the mean ± SEM *versus* control. The value of the protein expression was quantified by ImageJ program, and the labeling intensity of the different targets observed in the tumors was obtained using Student's *t*-test (*p < 0.05, **p < 0.01, ***p < 0.001). Magnification of 40×. Bar: 20 μm. (Up miR-152-3p: Up-regulated miR-152-3p.)

melatonin through MT1 receptor [42].

The protective action of melatonin has already been studied, and our research group has identified that in concentrations higher than the physiological level, melatonin can have antitumor actions, such as decreased cell proliferation, angiogenesis and metastasis in MDA-MB-231 and MCF-7 cells [17,28–30]. In this present study, melatonin decreases the cell viability of the MDA-MB-468 cell line at 1 mM concentration. Corroborating our results, other studies have shown that 1 mM was sufficient to decrease the proliferation of MDA-MB-231 cells [17,43] and in contrast, this hormone was not able to decrease the process in MDA-MB-435, highly metastatic cells [43]. However, as there are no other studies using pharmacological doses in the MDA-MB-468 cells, our result is an original contribution, and this result of 1 mM dose was used for all experiments. This defines a possible use of this molecule as adjuvant in triple-negative breast cancer therapy by using pharmacological concentration (higher than physiological concentrations). However, as this is an experimental research, translational studies are needed to understand an equivalent concentration to become clinically relevant for treating patients in the future.

We found that miRNAs are modulated by melatonin in the MDA-MB-468 cell line. Overall, melatonin increased the expression of miRNAs which are poorly expressed and those that the hormone decreased are strongly expressed. Melatonin increased the miRNAs considered to be tumor suppressors let-7c, miR-152, miR-214, miR-29b, and also decreased those considered oncomiRs, miR-107, miR-10a, miR-15b, miR-20a, which are altered in mammary tumors. These miRNAs regulate the cell proliferation promoter/inhibitor genes, inducers of apoptosis, cell migration and metastasis, angiogenesis and tumorigenesis [4,24,44–49]. Despite the scarcity of work relating miRNAs and melatonin, our study corroborates literature, since this hormone has been shown to inhibit oncomiRs, such as miR-155 in glioma cells [50] and miR-24 in colon and breast cancer cells (MCF-7) [51]. In these cases, this modulation caused a decrease in tumor growth, cell proliferation and invasion. A recent review study showed a lot of potential for this hormone in regulating non-coding RNAs, including miRNAs, once this hormone acts, down-regulating or over-expressing miRNAs involved with cancer, demonstrating a beneficial role in all the cases illustrated [52].

We note that melatonin was able to enhance the expression of mir-152 in the MDA-MB-468 cell line but did not modulate it in the MDA-MB-231 cell line, demonstrating that the miRNAs can be regulated in different ways in cell lines of the same phenotypic profile. This fact can be caused by the different genetic backgrounds of the cell lines, while MDA-MB-468 cells carry PTEN mutation [53], MDA-MB-231 cells contain KRas mutation [54]. Thus, more studies are necessary to identify these backgrounds and their relation with miRNAs. We also identified that melatonin increases this miRNA *in vivo*, after implantation of MDA-MB-468 cells in BALB/c mice. Decreased miR-152 has been associated with the process of cell proliferation, invasion and angiogenesis in different neoplasms such as breast [55], hepatocarcinoma [56], ovary [57] and gastric cancer [58]. The high expression of miR-152-3p is related to the inhibition of IGF-1R expression through its binding to the 3′-UTR region, leading to the blockade of HIF-1α and VEGF expression [9]. Because of the key role of IGF-1R, HIF-1α and VEGF in the angiogenesis process, and as they were already identified to be targets of miR-152-3p, we chose them to verify the action of melatonin. These targets are highly expressed in TNBC compared to non-TNBC tumors [59].

We found an increase in the gene expression of IGF-1R, HIF-1α and

VEGF caused by melatonin and the up-regulated miR-152-3p, but this hormone also decreases the protein expression of the target genes. Thus suggesting a post-transcriptional function of melatonin, and confirming the action of this miRNA. Our results corroborate those of Xu and collaborators [9], since the high expression of miR-152 in MDA-MB-468 cells led to a decrease in the protein expression of the IGF-1R, HIF-1α and VEGF genes. The authors demonstrated the increase of these genes by miR-152 in MCF-10a, MCF-7, T47D and MDA-MB-231 cells. Although this miRNA is described as a tumor suppressor in different tumor cell lines [24], its action on the MDA-MB-468 cells is unprecedented. Several studies attribute to melatonin the inhibition of angiogenesis by reducing the genes related to this process. Others studies by our group described how melatonin acts on genes related to angiogenesis, such as HIF-1α and VEGF in breast cancer cells MCF-7 and MDA-MB-231 under hypoxic conditions [29]. In another study, our group identified that the hormone increases IGF-1R expression in MDA-MB-231 when related to the apoptotic process [30]. However, there are no studies on the modulation of melatonin in IGF-1R and angiogenesis. Activation of the IGF-1R signalling pathway promotes the proliferation, survival, and metastasis of breast cancer cells. When this receptor is blocked, it may also block the expression of HIF-1α and VEGF [9]. Under conditions of hypoxia, HIF-1α is stimulated and its degradation is blocked. It moves to the nucleus, where it will stimulate the expression of several genes that contribute to tumor progression, such as VEGF [60]. VEGF is widely produced in tumors, generating a vast and chaotic vascular network. This elevated expression is able to form new vessels in a quiescent vasculature, through an initial vasodilation, vascular permeability of pre-existing capillaries. The extravasation of plasma proteins occurs, establishing a matrix where the endothelial cells migrate [61].

5. Conclusions

In summary, our study for the first time demonstrated that melatonin increases miR-152-3p and decreases the protein expression of IGF-1R, HIF-1α and VEGF in MDA-MB-468 cells and MDA-MB-468 cell line derived xenograft, suggesting a post-transcriptional action of this hormone. Our results contribute to the understanding of the action of melatonin in triple negative breast cancer and in the miR-152/IGF-IR, HIF-1α, VEGF pathway. This pathway is well known as a contributor to angiogenesis, so melatonin appears as a molecule for adjuvant therapeutic use against this important tumor process.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

References

[1] H.A. Hagrass, S. Sharaf, H.F. Pasha, E.A. Tantawy, R.H. Mohamed, R. Kassem, Circulating microRNAs - a new horizon in molecular diagnosis of breast cancer, Genes Cancer 6 (5–6) (2015) 281–287, https://doi.org/10.18632/genesandcancer.66.

[2] A. McGuire, J.A.L. Brown, M.J. Kerin, Metastatic breast cancer: the potential of miRNA for diagnosis and treatment monitoring, Cancer Metastasis Rev. 34 (1) (2015) 145–155, https://doi.org/10.1007/s10555-015-9551-7.

[3] Q. Xu, L.-Z. Liu, Y. Yin, et al., Regulatory circuit of PKM2/NF-κB/miR-148a/152-modulated tumor angiogenesis and cancer progression, Oncogene 34 (43) (2015) 5482–5493, https://doi.org/10.1038/onc.2015.6.

[4] W. Wang, Y. Luo, MicroRNAs in breast cancer: oncogene and tumor suppressors with clinical potential, J. Zhejiang Univ. B 16 (1) (2015) 18–31, https://doi.org/10.1631/jzus.B1400184.

[5] J. Castañeda-Gill, J. Vishwanatha, Antiangiogenic mechanisms and factors in breast cancer treatment, J. Carcinog. 15 (1) (2016) 1, https://doi.org/10.4103/1477-3163.176223.

[6] C. Madu, L. Li, Y. Lu, Selection, analysis and improvement of anti-angiogenesis compounds identified by an anti-HIF-1α screening and validation system, J. Cancer 7 (14) (2016) 1926–1938, https://doi.org/10.7150/jca.15603.

[7] G. Danza, C. Di Serio, F. Rosati, et al., Notch signalling modulates hypoxiainduced neuroendocrine, 10 (2) (2015) 230–238, https://doi.org/10.1158/1541.

[8] Y. Xu, B. Chen, S.K. George, B. Liu, Downregulation of microRNA-152 contributes to high expression of DKK1 in multiple myeloma, RNA Biol. 12 (12) (2015) 1314–1322, https://doi.org/10.1080/15476286.2015.1094600.

[9] Q. Xu, Y. Jiang, Y. Yin, et al., A regulatory circuit of miR-148 a/152 and DNMT 1 in modulating cell transformation and tumor angiogenesis through IGF-IR and IRS 1, J. Mol. Cell Biol. 5 (2013) 3–13.

[10] R. Girgert, V. Hanf, G. Emons, C. Gründker, Membrane-bound melatonin receptor MT1 down-regulates estrogen responsive genes in breast cancer cells, J. Pineal Res. 47 (1) (2009) 23–31, https://doi.org/10.1111/j.1600-079X.2009.00684.x.

[11] A. Cucina, S. Proietti, F. D'Anselmi, et al., Evidence for a biphasic apoptotic pathway induced by melatonin in MCF-7 breast cancer cells, J. Pineal Res. 46 (2) (2009) 172–180, https://doi.org/10.1111/j.1600-079X.2008.00645.x.

[12] C. Martínez-Campa, A. González, M.D. Mediavilla, et al., Melatonin inhibits aromatase promoter expression by regulating cyclooxygenases expression and activity in breast cancer cells, Br. J. Cancer 101 (9) (2009) 1613–1619, https://doi.org/10.1038/sj.bjc.6605334.

[13] S.M. Hill, D.E. Blask, S. Xiang, et al., Melatonin and associated signaling pathways that control normal breast epithelium and breast cancer, J. Mammary Gland Biol. Neoplasia 16 (3) (2011) 235–245, https://doi.org/10.1007/s10911-011-9222-4.

[14] S.-C. Su, M.-J. Hsieh, W.-E. Yang, W.-H. Chung, R.J. Reiter, S.-F. Yang, Cancer metastasis: mechanisms of inhibition by melatonin, J. Pineal Res. 62 (1) (2017) e12370, , https://doi.org/10.1111/jpi.12370.

[15] C. Li, G. Li, D.X. Tan, F. Li, X. Ma, A novel enzyme-dependent melatonin metabolite in humans, J. Pineal Res. 54 (1) (2013) 100–106, https://doi.org/10.1111/jpi.12003.

[16] V. Alvarez-García, A. González, C. Alonso-González, C. Martínez-Campa, S. Cos, Antiangiogenic effects of melatonin in endothelial cell cultures, Microvasc. Res. 87 (2013) 25–33, https://doi.org/10.1016/j.mvr.2013.02.008.

[17] B.V. Jardim-Perassi, A.S. Arbab, L.C. Ferreira, et al., Effect of melatonin on tumor growth and angiogenesis in xenograft model of breast cancer, PLoS One 9 (1) (2014), https://doi.org/10.1371/journal.pone.0085311.

[18] R.C. Lee, R.L. Feinbaum, V. Ambros, The *C. elegans*\rheterochronic gene lin-4 encodes small RNAs with antisense\rcomplementarity to lin-14, Cell 75 (843–85) (1993) 843–854, https://doi.org/10.1016/0092-8674(93)90529-Y.

[19] G.A. Calin, M. Ferracin, A. Cimmino, et al., A microRNA signature associated with prognosis and progression in chronic lymphocytic leukemia, N. Engl. J. Med. 353 (17) (2005) 1793–1801, https://doi.org/10.1056/NEJMoa050995.

[20] K.R. Kutanzi, O.V. Yurchenko, F.A. Beland, V.F. Checkhun, I.P. Pogribny, MicroRNA-mediated drug resistance in breast cancer, Clin. Epigenetics 2 (2) (2011) 171–185, https://doi.org/10.1007/s13148-011-0040-8.

[21] E. O'Day, A. Lal, MicroRNAs and their target gene networks in breast cancer, Breast Cancer Res. 12 (2) (2010) 201, https://doi.org/10.1186/bcr2484.

[22] G. Bertoli, C. Cava, I. Castiglioni, MicroRNAs: new biomarkers for diagnosis, prognosis, therapy prediction and therapeutic tools for breast cancer, Theranostics 5 (10) (2015) 1122–1143, https://doi.org/10.7150/thno.11543.

[23] D. Sengupta, M. Deb, S.K. Rath, et al., DNA methylation and not H3K4 trimethylation dictates the expression status of miR-152 gene which inhibits migration of breast cancer cells via DNMT1/CDH1 loop, Exp. Cell Res. 346 (2) (2016) 176–187, https://doi.org/10.1016/j.yexcr.2016.07.023.

[24] X. Liu, J. Li, F. Qin, S. Dai, MiR-152 as a tumor suppressor microRNA: target recognition and regulation in cancer, Oncol. Lett. 11 (6) (2016) 3911–3916, https://doi.org/10.3892/ol.2016.4509.

[25] R. Reiter, S. Rosales-Corral, D.-X. Tan, et al., Melatonin, a full service anti-cancer agent: inhibition of initiation, progression and metastasis, Int. J. Mol. Sci. 18 (4) (2017) 843, https://doi.org/10.3390/ijms18040843.

[26] V. Alvarez-García, A. González, C. Alonso-González, C. Martínez-Campa, S. Cos, Regulation of vascular endothelial growth factor by melatonin in human breast cancer cells, J. Pineal Res. 54 (4) (2013) 373–380, https://doi.org/10.1111/jpi.12007.

[27] A. González-González, A. González, C. Alonso-González, J. Menéndez-Menéndez, C. Martínez-Campa, S. Cos, Complementary actions of melatonin on angiogenic factors, the angiopoietin/Tie2 axis and VEGF, in co-cultures of human endothelial and breast cancer cells, Oncol. Rep. 39 (1) (2017) 433–441, https://doi.org/10.3892/or.2017.6070.

[28] B. Maschio-Signorini L, B. Gelaleti G, G. Moschetta M, et al., Melatonin regulates angiogenic and inflammatory proteins in MDA-MB-231 cell line and in co-culture with cancer-associated fibroblasts, Anti Cancer Agents Med. Chem. 16 (11) (2016) 1474–1484, https://doi.org/10.2174/1871520616666160422105920.

[29] B.V. Jardim-Perassi, M.R. Lourenço, G.M. Doho, et al., Melatonin regulates angiogenic factors under hypoxia in breast Cancer cell lines, Anti Cancer Agents Med. Chem. 16 (3) (2016) 347–358 http://www.ncbi.nlm.nih.gov/pubmed/25963143 , Accessed date: 9 November 2017.

[30] G.B. Gelaleti, T.F. Borin, L.B. Maschio-Signorini, et al., Efficacy of melatonin, IL-25 and siIL-17B in tumorigenesis-associated properties of breast cancer cell lines, Life Sci. 183 (2017) 98–109, https://doi.org/10.1016/j.lfs.2017.06.013.

[31] S.K. Shenouda, S.K. Alahari, MicroRNA function in cancer: oncogene or a tumor suppressor? Cancer Metastasis Rev. 28 (3–4) (2009) 369–378, https://doi.org/10.1007/s10555-009-9188-5.

[32] N.H. Goradel, M.H. Asghari, M. Moloudizargari, B. Negahdari, H. Haghi-Aminjan, M. Abdollahi, Melatonin as an angiogenesis inhibitor to combat cancer: mechanistic evidence, Toxicol. Appl. Pharmacol. 335 (2017) 56–63, https://doi.org/10.1016/j.taap.2017.09.022.

[33] K. Seiffert, B. Schmalfeldt, V. Müller, N. Therapieansätze, D. Subtypen, Hormon- ZT. Zielgerichtete Therapie des Mammakarzinoms Einführung Zielgerichtete Therapie des HER2-, Dtsch. Med. Wochenschr. 142 (22) (2017) 1669–1675, https://doi.org/10.1055/S-0043-108468.

[34] I. Azimi, R.M. Petersen, E.W. Thompson, S.J. Roberts-Thomson, G.R. Monteith, Hypoxia-induced Reactive Oxygen Species Mediate N-cadherin and SERPINE1 Expression, EGFR Signalling and Motility in MDA-MB-468 Breast Cancer Cells, (2017), pp. 1–11, https://doi.org/10.1038/s41598-017-15474-7 (October 2016).

[35] P. Garbati, S. Ravera, S. Scarfì, et al., Effects on energy metabolism of two guanidine molecules, (Boc)2-creatine and metformin, J. Cell. Biochem. 118 (9) (2017) 2700–2711, https://doi.org/10.1002/jcb.25914.

[36] L. Wei, K. Li, X. Pang, et al., Leptin promotes epithelial-mesenchymal transition of breast cancer via the upregulation of pyruvate kinase M2, J. Exp. Clin. Cancer Res. 35 (1) (2016) 166, https://doi.org/10.1186/s13046-016-0446-4.

[37] L.C. Chang, Y.L. Yu, M.T. Hsieh, et al., A novel microtubule inhibitor, MT3-037, causes cancer cell apoptosis by inducing mitotic arrest and interfering with microtubule dynamics, Am. J. Cancer Res. 6 (4) (2016) 747–763.

[38] E. Chinchar, K.L. Makey, J. Gibson, et al., Sunitinib significantly suppresses the proliferation, migration, apoptosis resistance, tumor angiogenesis and growth of triple-negative breast cancers but increases breast cancer stem cells, Vasc. Cell 6 (2014) 12, https://doi.org/10.1186/2045-824X-6-12.

[39] Y. Li, S. Li, Y. Zhou, et al., Melatonin for the prevention and treatment of cancer, Oncotarget 8 (24) (2017) 39896–39921, https://doi.org/10.18632/oncotarget.16379.

[40] S.M. Hill, L.L. Spriggs, M.A. Simon, H. Muraoka, D.E. Blask, The growth inhibitory action of melatonin on human breast cancer cells is linked to the estrogen response system, Cancer Lett. 64 (3) (1992) 249–256 http://www.ncbi.nlm.nih.gov/pubmed/1638517 , Accessed date: 6 January 2018.

[41] D. Hevia, P. González-Menéndez, I. Quiros-González, et al., Melatonin uptake through glucose transporters: a new target for melatonin inhibition of cancer, J. Pineal Res. 58 (2) (2015) 234–250, https://doi.org/10.1111/jpi.12210.

[42] L. Mao, L. Yuan, S. Xiang, et al., Molecular deficiency (ies) in MT₁ melatonin signaling pathway underlies the melatonin-unresponsive phenotype in MDA-MB-231 human breast cancer cells, J. Pineal Res. 56 (3) (2014) 246–253, https://doi.org/10.1111/jpi.12117.

[43] E.S. Leman, B.F. Sisken, S. Zimmer, K.W. Anderson, Studies of the interactions between melatonin and 2 Hz, 0.3 mT PEMF on the proliferation and invasion of human breast cancer cells, Bioelectromagnetics 22 (3) (2001) 178–184 http://www.ncbi.nlm.nih.gov/pubmed/11255213 , Accessed date: 6 January 2018.

[44] H.B. Han, J. Gu, H.J. Zuo, et al., Let-7c functions as a metastasis suppressor by targeting MMP11 and PBX3 in colorectal cancer, J. Pathol. 226 (3) (2012) 544–555, https://doi.org/10.1002/path.3014.

[45] S.-J. Yi, L.-L. Li, W.-B. Tu, MiR-214 negatively regulates proliferation and WNT/β-catenin signaling in breast cancer, Eur. Rev. Med. Pharmacol. Sci. 20 (24) (2016) 5148–5154 http://www.ncbi.nlm.nih.gov/pubmed/28051254 , Accessed date: 13 November 2017.

[46] Y. Li, B. Cai, L. Shen, et al., MiRNA-29b suppresses tumor growth through simultaneously inhibiting angiogenesis and tumorigenesis by targeting Akt3, Cancer Lett. 397 (2) (2017) 111–119, https://doi.org/10.1016/j.canlet.2017.03.032.

[47] L. Zhang, P. Ma, L. Sun, et al., MiR-107 down-regulates SIAH1 expression in human breast cancer cells and silencing of miR-107 inhibits tumor growth in a nude mouse model of triple-negative breast cancer, Mol. Carcinog. 55 (5) (2016) 768–777, https://doi.org/10.1002/mc.22320.

[48] M. Kedmi, N. Ben-Chetrit, C. Körner, et al., EGF induces microRNAs that target suppressors of cell migration: miR-15b targets MTSS1 in breast cancer, Sci. Signal. 8 (368) (2015) ra29, https://doi.org/10.1126/scisignal.2005866.

[49] L. Liu, J. He, X. Wei, et al., MicroRNA-20a-mediated loss of autophagy contributes to breast tumorigenesis by promoting genomic damage and instability, Oncogene (1) (2017) 5874–5884, https://doi.org/10.1038/onc.2017.193.

[50] J. Gu, Z. Lu, C. Ji, et al., Melatonin inhibits proliferation and invasion via repression of miRNA-155 in glioma cells, Biomed Pharmacother 93 (2017) 969–975, https://doi.org/10.1016/j.biopha.2017.07.010.

[51] F. Mori, M. Ferraiuolo, R. Santoro, et al., Multitargeting activity of miR-24 inhibits long-term melatonin anticancer effects, Oncotarget 7 (15) (2016) 20532–20548, https://doi.org/10.18632/oncotarget.7978.

[52] S.-C. Su, R.J. Reiter, H.-Y. Hsiao, W.-H. Chung, S.-F. Yang, Functional interaction

between melatonin signaling and noncoding RNAs, Trends Endocrinol. Metab. 29 (6) (2018) 435–445, https://doi.org/10.1016/j.tem.2018.03.008.

[53] S.B. Turturro, M.S. Najor, C.E. Ruby, M.A. Cobleigh, A.M. Abukhdeir, Mutations in PIK3CA sensitize breast cancer cells to physiologic levels of aspirin, Breast Cancer Res. Treat. 156 (1) (2016) 33–43, https://doi.org/10.1007/s10549-016-3729-8.

[54] A. Hollestelle, F. Elstrodt, J.H.A. Nagel, W.W. Kallemeijn, M. Schutte, Phosphatidylinositol-3-OH kinase or RAS pathway mutations in human breast cancer cell lines, Mol. Cancer Res. 5 (2) (2007) 195–201, https://doi.org/10.1158/1541-7786.MCR-06-0263.

[55] A. Maimaitiming, A. Wusiman, A. Aimudula, T. Tudahong, D. Aisimutula, Downregulation of microRNA-152 and inhibition of cell proliferation, migration, and invasion in breast cancer, Oncol. Res. Featuring Preclinical Clin. Cancer Ther. (2017), https://doi.org/10.3727/096504017X14974821032421.

[56] H. Zhang, Y. Li, M. Lai, The microRNA network and tumor metastasis, Oncogene 29 (7) (2010) 937–948, https://doi.org/10.1038/onc.2009.406.

[57] X. Zhou, Z.-N. Wang, F. Zhao, et al., Altered expression of miR-152 and miR-148a in ovarian cancer is related to cell proliferation, Oncol. Rep. 27 (2) (2012) 447–454, https://doi.org/10.3892/or.2011.1482.

[58] R. Zhai, X. Kan, B. Wang, et al., miR-152 suppresses gastric cancer cell proliferation and motility by targeting CD151, Tumor Biol. 35 (11) (2014) 11367–11373, https://doi.org/10.1007/s13277-014-2471-2.

[59] A. Bahnassy, M. Mohanad, M.F. Ismail, S. Shaarawy, A. El-Bastawisy, A.R.N. Zekri, Molecular biomarkers for prediction of response to treatment and survival in triple negative breast cancer patients from Egypt, Exp. Mol. Pathol. 99 (2) (2015) 303–311, https://doi.org/10.1016/j.yexmp.2015.07.014.

[60] J. Vriend, R.J. Reiter, Melatonin and the von Hippel-Lindau/HIF-1 oxygen sensing mechanism: a review, Biochim. Biophys. Acta Rev. Cancer 1865 (2) (2016) 176–183, https://doi.org/10.1016/j.bbcan.2016.02.004.

[61] G. Bergers, L.E. Benjamin, Angiogenesis: tumorigenesis and the angiogenic switch, Nat. Rev. Cancer 3 (6) (2003) 401–410, https://doi.org/10.1038/nrc1093.