The Effect Of Malignant Breast Tissue Stromal Cells On The Growth Of Cancer Cells And The Relationship Of miRNA

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Research Article

Abstract

Purpose

Inflammatory signals secreted from the tumor microenvironment are thought to promote tumor growth and survival. It has been reported that stromal cells in the tumor microenvironment have similar characteristics to tumor-associated cells. In addition to miRNAs play critical roles in various diseases, including cancer. In this study, we aimed to investigate the effects of co-culture of cancer cells and stromal cells isolated from normal and malignant breast tissue on each other and the possible effects of miRNAs on these interactions.

Methods

The characterized stromal cells were co-cultured with an MDA-MB-231 cancer cell line. The proliferation capacity of the experimental groups was evaluated using the WST-1 assay. The expression of breast cancer-specific miRNAs and related genes were assessed by real-time PCR. ELISA assay was performed to determine the concentration of some cytokines and chemokines.

Results

We found that the microenvironment plays an important role in the development of cancer, confirming the changes in the expression of oncogenic and tumor-suppressor miRNA and their target genes after co-culture with malignant stromal cells.

Conclusion

As a result of the studies, specific gene expressions of related signaling pathways were detected in correlation with miRNA changes and the effects of tumor microenvironment on tumorigenesis were revealed in detail. miRNAs have been shown to play an important role in cancer development in recent studies. The idea that these small molecules can be used in diagnosis and treatment is becoming stronger day by day. We believe that new treatment approaches involving the tumor microenvironment and using miRNAs as markers are promising.

Introduction

MicroRNAs (miRNAs), which regulate gene and protein expression through the mechanism of RNA interference (RNAi), are non-protein-coding, single-stranded RNAs of 18-24 nucleotides in length. MiRNAs, which play a role in regulating gene expression, are synthesized from DNA as primary transcripts (pri-miRNA) by the enzyme RNA polymerase II. Primary transcripts, are processed into short stem-loop structures, called pre-miRNA, and then into functional miRNA. Pre-miRNAs are converted into mature

miRNAs by interaction with the Dicer endonuclease in the cytoplasm. Dicer also initiates the formation of the RNA-induced silencing complex (RISC). This complex is responsible for miRNA expression and gene silencing. In summary, mature miRNAs contribute to the regulation of protein synthesis by reducing the expression of target genes. [1–11].

miRNAs are involved in a variety of biological functions, including cell proliferation, differentiation, and apoptosis. Therefore, they play important roles in various diseases, including cancer. It has been observed that more than half of miRNA genes are localized in cancer-relevant or more sensitive gene regions, and these localization features are thought to play an important role in the pathogenesis of neoplasia. [5, 8, 10, 12–17].

Most miRNAs that can function as oncogenes or tumor suppressors are known to play important roles in carcinogenesis. The first evidence for this was provided by a study conducted in patients with Chronic Lymphocytic Leukemia (CLL). In the following years, altered miRNA levels were found in various cancers, including breast cancer, and the comparison of their levels in cancer cells with normal cells gained importance for the diagnosis and treatment of cancer. [2, 4, 8–12, 18, 19].

In the results of the studies conducted in recent years, it was found that the regulatory role of miRNAs in the process of carcinogenesis is not limited to cancer cells, but is also related to the tumor stroma [10, 20, 21]. The solid tumor mass is composed of a complex mixture that includes the tumor microenvironment. The tumor microenvironment consists of fibroblasts, macrophages, endothelial cells, leukocytes, and extracellular matrix and soluble factors [21–24]. In addition, normal cells, including inflammatory cells, tumor-associated fibroblasts (TAF), and tumor-associated stromal cells are also present in the inflammatory tumor microenvironment [21, 25–27]. Studies have shown that these cells in the tumor microenvironment, along with tumor cells, also influence tumor behavior [26–28]. However, inflammatory signaling molecules secreted by the tumor microenvironment are thought to promote tumor growth and persistence. Studies conducted in this context have reported that some proinflammatory cytokines derived from stromal cells in the tumor microenvironment stimulate tumor cell proliferation by acting through various signaling pathways, cited [9, 22, 25, 26, 29].

Mesenchymal Stem Cells (MSCs), which are thought to be found in the tumor microenvironment, are defined as cells that have a strong self-renewal capacity and can differentiate into other cells from cells in the tissue from which they originate. These cells are often referred to as stromal cells. In addition to their regenerative properties in damaged and inflamed areas, some studies have reported that stromal cells acquire similar properties to tumor-associated cells in the tumor microenvironment [22, 29, 30–33].

The functions of miRNAs need to be studied in detail to better understand the molecular basis of cancer, discover new markers, determine prognosis, and consequently develop new treatment approaches. For this reason, we wanted to investigate how cancer cells and stromal cells from healthy and malignant breast tissue influence each other in their co-cultures and what role miRNAs play in these possible effects.

Materials And Methods

Cell lines and isolation methods

Human Healthy Breast Tissue-Mesenchymal Stem Cells (hHBT-MSCs) were obtained from breast reduction surgeries, whereas Human Malignant Breast Tissue-Mesenchymal Stem Cells (hMBT-MSCs) were obtained from tumor removal surgeries. In a sterile environment (class II "Laminar Cabinet), breast tissues from healthy and cancer patients were placed in a petri dish and washed with 5% antibiotic Hank's Buffered Salt Solution (HBSS) (Gibco Invitrogen, Grand Island, USA) and cut into small pieces with scissors and treated with the enzyme collagenase type II (Gibco Invitrogen, Grand Island, USA). After washing, the supernatant was removed and the cells were cultured with DMEM/F12 (Hyclone, USA) medium containing Fetal Bovine Serum (FBS), antibiotics, insulin and hydrocortisone at 37°C in a humid atmosphere with 5% CO2. On the third day, non-adherent cells were removed and fresh medium was added. When the bottom of the flask was approximately 70% covered with cells, passage was performed. At the third passage (P3), characterization of the stromal cells was performed.

The breast cancer cell line MDA-MB-231 (ATCC) was also grown and propagated in medium enriched with serum, antibiotics, insulin and hydrocortisone (DMEM / F12), which showed good proliferation.

Characterization methods of mesenchymal stromal cells

Flow cytometry analysis

Flow cytometric analysis of the cells after P3 was performed using FACS Calibur flow cytometry instrument. The mesenchymal stromal cells were harvested by trypsinization, homogenized approximately 5×10^6 cells/ml in PBS and probed with fluorescein isothiocyanate (FITC) and phycoerythrin (PE)-conjugated monoclonal antibodies and corresponding isotype controls for specific cell surface markers. The prepared cell suspension was read using FACS Calibur (BD Biosciences, San Diego, USA) flow cytometry instrument and analyzed using BD CellQuest Software (BD Pharmingen).

In vitro differentiation studies of mesenchymal stromal cells (Adipogenic, osteogenic and neurogenic differentiation)

To induce adipogenic differentiation, cells of P3 were seeded in six-well plates. LDMEM adipogenic medium (Invitrogen/Gibco, Paisley, UK) was used, supplemented with 10% FBS (Gibco Invitrogen, Grand Island, USA), 200 μM indomethacin (Sigma-Aldrich, St. Louis, MO, USA), 10 μg/ml insulin (Invitrogen/Gibco, Paisley, UK), 0.5 mM isobutyl methylxanthine (IBMX- Sigma-Aldrich, St. Louis, MO, USA), 10^{-6} M dexamethasone (Sigma-Aldrich, Fluka Chemie AG, Buchs, Switzerland), and primocin (0.2%; InvivoGen, San Diego, CA, USA) for 15 days. As a control, cells were simultaneously seeded and cultured with mesenchymal stromal cell growth medium. The presence of lipid droplets was stained with the dye Oil Red O (Sigma-Aldrich, St. Louis, MO, USA) to determine adipogenic differentiation.

For osteogenic differentiation, cells were seeded in plates with six wells and cultured for 4 weeks using 'MesenCult Osteogenic Differentiation Kit'. After 4 weeks, osteogenic differentiation was detected by histological staining Alizarin Red S. As a control, cells were simultaneously seeded and cultured with mesenchymal stromal cell growth medium. The presence of osteogenic differentiation after fixation was detected by Alizarin Red S staining of calcium nodules.

To induce neurogenic differentiation, cells from were seeded in six-well plates. When cells reached appropriate confluence, they were incubated for 24-72 hours in LDMEM, a neurogenic culture medium containing 10% FBS, 10 ng/ml FGF, 10 ng/ml EGF; Epidermal Growth Factor, 10 ng/ml BDNF; Brain-derived neurotrophic factor, 0.5 mM isobutyl methylxanthine and 0.2% primocin. As a control, cells were simultaneously seeded and cultured with mesenchymal stromal cell growth medium.

Transswell co-culture experiments

In our study, an indirect co-culture technique based on paracrine interaction was performed by preventing two different cell sources from contacting each other using membranes with different sized pores called insert (intermediate). After refreshing MDA-MB-231 cells, 0.5×10^5 cells were seeded on six-well plates and 1×10^5 stromal cells from P3 were seeded on a semipermeable membrane and waited for 24 hours to generate adherent monolayers. At the end of this period, the semipermeable membranes were placed on the six-well plates and the co-culture process was continued for 4 days. To understand the interactions of the co-cultured cells, cancer cells and stromal cells were cultured separately in a 6-well culture plate as a control. Therefore, in our experience, 5 groups were used: Stromal cells obtained from healthy and malignant breast tissues, and experimental groups in which cancer lineage cells were cultured separately, as well as experimental groups in which co-cultures of stromal cells and cancer lineage cells were performed.

Water-Soluble Tetrazolium monosodium salt-1 (WST-1) cell viability and proliferation test

After co-culture, the water-soluble tetrazolium 1 (Cell Proliferation Reagent, WST-1) solution (Roche Diagnostics, Mannheim, Germany) was used to show the proliferation and cell viability of the stromal cells and MDA-MB-231 cancer cell line. Cells were seeded in a 12-well culture plate (25×10^3 stromal cells: 12.5×10^3 cancer cells) according to the designated experimental design and cultured for the time specified for co-culture. After this time, the medium was replaced with medium containing 10% WST-1 solution and incubated for 4 hours at 37°C in a humid atmosphere with 5% CO2. After incubation, the tetrazolium ring was evaluated by dehydrogenase enzymes in the cell mitochondria. The optical densities (OD) of the samples were then evaluated using a microplate reader (Versamax, USA) at 480 nm.

ELISA (Enzyme-Linked Immunosorbent Assay) method

The concentrations of IL-8 and IL-6 released into the supernatant after co-culture were determined in the monochromator system microplate reader (VersaMax, Molecular Device, USA) following the manufacturer's indicated procedural steps (Invitrogen, Camarillo, CA, USA).

Real-time polymerase chain reaction

After co-culture, cell pellets were homogenized in PBS and total RNA was isolated using High Pure RNA Isolation Kit (Roche, Mannheim, Germany). After isolation, RNA concentrations were measured using a Picodrop spectrophotometer. One microgram of total RNA was transcribed into complementary DNA (cDNA) using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Mannheim, Germany).

Amplification of target genes was performed with an equal amount of cDNA in the LightCycler 480 (RocheDiagnostics, Rotkreuz, Switzerland) real-time PCR instrument using appropriate Universal Probe Library (UPL) probes (Taqman Hydrolysis Probes). The target gene and the corresponding selected reference genes (HPRT) were amplified in the same wells using the appropriate primers and probes. Primers and probes were standardized as before and used as recommended by the manufacturer. PCR conditions were as follows: Incubation for 10 minutes at 95°C, followed by 45 cycles of 10 seconds denaturation at 95°C, 30 seconds annealing at 60°C and 1 second extension at 72°C. Results were analyzed using LightCycler software (version 4) using standards prepared at known concentrations by serial dilution. PCR activity was calculated using LightCycler software and only values between 1.85 and 2.0 were used.

Determination of miRNA expression levels

Pre-made plates called 'Pick-&-Mix microRNA PCR Panels' of the miRCURY LNATM Universal RT microRNA PCR System (EXIQON, United States) were used to determine miRNA expression levels, and the desired miRNAs were placed on them before the study. The system used consists of a two-step protocol.

1. cDNA synthesis
2. real-time PCR amplification.

Statistical Method

At least 3 replicates were performed for each experiment. The data of the studies were reported considering the standard deviation (±SD). All statistical analyzes were performed using SPSS 20.0 and data were analyzed using the Kruskal-Wallis and Independent Sample T-test. For the differences between the experimental and control groups, $p < 0.05$ was accepted as the statistical significance threshold.

Results

Isolation and Culture of Mesenchymal Stromal Cells

The tissue fragments removed from the operated cases were delivered to the laboratory, and the mesenchymal stromal cells were isolated by the enzymatic digestion method, following the appropriate procedural steps.

The isolated cells and the MDA-MB-231 breast cancer cell line were cultured under appropriate conditions and their morphological examination was performed by phase contrast microscopy. It was found that the mesenchymal stromal cells obtained from both healthy and malignant breast tissues were fibroblast-like and spindle-shaped in the first days of culture and proliferated by forming colonies in the following days (Fig. 1i-**ii**). However, it was observed that the breast cancer cell line had a cubic structure.

Characterization of Mesenchymal Stromal Cells

Characterization of mesenchymal stromal cells by flow cytometry

Flow cytometry analysis revealed that the stromal cells isolated from both healthy and malignant breast tissue were positive for CD105, CD29, CD73, CD44, CD90, CD71, CD10 and CD13. They were negative for CD34, CD33, CD45 and HLA G.

Characterization of mesenchymal stromal cells by *in vitro* differentiation studies

Osteogenic differentiation

Stromal cells obtained from healthy and malignant breast tissues for osteogenic differentiation were incubated in differentiation medium for approximately 4 weeks, and then the presence of calcium nodules was detected by Alizarin Red-S staining. In the groups that underwent bone differentiation in an osteogenic differentiation medium, calcified nodules were observed in our phase contrast microscopy studies before and after Alizarin Red-S staining. In the control groups where no differentiation medium was used, it was found that there were no calcium phosphate nodules and therefore no positive reaction with Alizarin Red dye.

Adipogenic differentiation

Morphological changes were observed after approximately 15 days in adipogenic differentiation medium used to transform stromal cells from healthy and malignant breast tissue into adipocytes. When the cells were fixed at the end of the 15-day incubation with the adipogenic differentiation medium and stained with the dye Oil Red O, it was observed that the oil vesicles formed in the experimental group showed a red color, while the cells in the control group showed no positive reaction with the dye Oil Red O.

Neurogenic differentiation

Stromal cells from healthy and malignant breast tissue were incubated in neurogenic differentiation medium for approximately 72 hours. Thereafter, the cells were observed to morphologically differentiate into neuron-like cells. No morphological changes were observed in the cells of the control group that were not treated with differentiation medium.

Evaluation of Co-Culture studies

Breast cancer cells and stromal cells obtained from healthy and malignant breast tissues were co-cultured through semipermeable membranes for 4 days, following characterization procedures. At the end of the 4-day incubation period, the WST-1 assay was performed to determine the proliferative capacity of the cells. Supernatants were collected for ELISA assays and RNA isolations were performed to determine the expression levels of the identified miRNA and associated genes.

WST-1 Test Evaluation

After the co-culture studies, the WST-1 assay was applied to detect the proliferation capacity of MDA-MB-231 cancer cells and mesenchymal stromal cells in the cancer microenvironment.

The analysis results of WST-1 assay revealed that the proliferation capacity of cancer cells increased significantly ($p \leq 0.001$) after co-culture with healthy and malignant stromal cells from breast tissue. However, it was found that the stromal cells derived from malignant breast tissue stimulated the proliferation of cancer cells more than the healthy cells ($p \leq 0.001$) (Fig. 2).

Evaluation of ELISA Results

To determine the concentrations of cytokines thought to be derived from stromal cells in the cancer microenvironment, the concentrations of IL-8 and IL-6 were determined by ELISA from supernatants obtained after co-culture of cancer cells with stromal cells from malignant and healthy breast tissue.

As a result of ELISA assay to determine the levels of IL-8, which is known to be associated with metastasis, the levels released by hHBT-MSCs ($p \leq 0.01$) and hMBT-MSCs ($p \leq 0.001$) were statistically significantly higher compared to the breast cancer cell line. However, a statistically significant ($p \leq 0.001$) increase was observed in both experimental groups after co-culturing hHBT-MSCs and hMBT-MSCs and cancer cells. It was also found that the amount of cancer cells was statistically significantly higher ($p \leq 0.001$) after co-culture with hMBT-MSCs than after co-culture with hHBT-MSCs (Fig. 3).

As a result of the ELISA assay to determine the IL-6 level, a significant ($p \leq 0.001$) increase in the level of cancer cells was found after co-culture with hHBT-MSCs and hMBT-MSCs. Moreover, it was found that IL-6 levels released by hHBT-MSCs ($p \leq 0.01$) and hMBT-MSCs ($p \leq 0.001$) were significantly higher than IL-6 levels released by cancer cells (Fig. 4).

Evaluation of gene and miRNA expression results

After co-culturing the breast cancer cell line with stromal cells from healthy and malignant breast tissues, breast cancer associated miRNAs (let7, miR-17-5p), as well as the expression levels of some genes (HMGA2, AIB1) of the pathways associated with these miRNAs were quantified in all experimental groups.

The result of gene and miRNA expression analysis was that the expression of let-7a, one of the miRNAs with tumor suppressor properties, significantly decreased after co-culture with hMBT-MSCs in cancer cells, although not statistically significant; on the contrary, an increase was observed after co-culture with

hHBT-MSCs. Moreover, the let-7a expression of hMBT-MSCs and hHBT-MSCs cultured alone was decreased compared to cancer cells, with a statistically significant (*: p≤0.05) decrease especially in hMBT-MSCs. Furthermore, when the expression levels were compared between the two groups after co-culturing with hMBT-MSCs and hHBT-MSCs of the cancer cell line, a statistically significant increase (*: p< 0.05) was observed in the experimental group co-cultured with hHBT-MSCs (Fig. 5).

The expression levels of HMGA2, which is a target of let-7 family members and is considered an oncogene, were determined in the opposite direction to let-7a. While an increase in HMGA2 levels was observed in cancer cells after co-culture with hMBT-MSCs, a decrease was observed after co-culture with hHBT-MSCs. Furthermore, when the expression levels of hMBT-MSCs and hHBT-MSCs cultured alone were compared, it was found that the expression was significantly higher in hMBT-MSCs (**: p≤0.01) (Fig. 5).

It was found that the expressions of miR-17-5p, which acts as a tumor suppressor, and the oncogenic gene AIB1, which is one of its targets, were opposite to each other, similar to the changes observed between let-7a and HMGA2. As a result of the analyzes performed, although the expression of miR-17-5p was not statistically significant, it was found to decrease in cancer cells after co-cultures with hMBT-MSCs and to increase after co-cultures with hHBT-MSCs. Moreover, when mir-17-5p expressions were compared between the two groups after co-culture with hMBT-MSCs and hHBT-MSCs, a statistically significant (**: p≤0.01) increase was observed in the experimental group in which hHBT-MSCs were co-cultured. When the mir-17-5p expression levels of hMBT-MSCs and hHBT-MSCs were compared with each other, a significant (*: p≤0.05) increase was also observed in hHBT-MSCs (Fig. 6).

It was observed that the expression levels of the gene with oncogenic function AIB1, which was indicated as one of the targets of miR-17-5p, decreased or increased in the opposite direction of miR-17-5p. It was found that AIB1 expressions of cancer cells significantly increased after co-culture with hMBT-MSCs (**: p≤0.01). In addition, AIB1 expression levels in hMBT-MSCs cultured alone were also higher compared to cancer cells, although not statistically significant. When AIB1 expression levels were compared between hMBT-MSCs and hHBT-MSCs after co-culture between the two groups, a significant (*: p≤0.05) decrease was observed in the experimental group in which hHBT-MSCs were co-cultured (Fig. 6).

Discussion

Cancer, one of the most important health problems of today, has been one of the most frequent research topics in the history of medicine. Breast cancer is the most common and constantly increasing cancer in women [21, 34]. Molecular studies have become more important due to recent developments in the diagnosis, staging, treatment and survival of breast cancer. However, further studies are needed to detect breast cancer at an early stage, fully understand the disease, determine prognostic factors, and develop targeted treatment approaches that incorporate the tumor microenvironment.

The tumor microenvironment consists of tumor cells and immune, stromal, and inflammatory cells. It is claimed that cytokines, growth factors, and adhesion molecules released by these cells can promote

tumor progression and metastasis. All of these cells are closely associated with each other, play an important role in inflammatory and pro-angiogenic processes, and support tumor cell proliferation [21, 24, 32, 33, 35].

After studies found that cells derived from healthy and malignant breast tissues had MSC properties, these cells were co-cultured with breast cancer cells. As a result of the WST-1 assay performed to show the proliferation capacity of stromal cells and MDA-MB-231 cancer cells after co-culture studies, it was found that stromal cells increased the proliferation capacity of cancer cells. It was found that stromal cells derived from malignant breast tissue increased the proliferation capacity of cancer cells more significantly than healthy stromal cells. This situation led us to believe that stromal cells in the tumor microenvironment are more prone to adopt malignant characteristics, and that some factors released by stromal cells as a result of their interaction with cancer cells may cause tumor cell development and progression. Similar to our study, some studies reported that there are findings related to tumor cell proliferation and increased metastatic ability after co-culture with MSCs [36]. Moreover, Karnoub et al. (2007) showed in their study that stem cells support proliferation and metastasis of breast tumor cells and that bone marrow-derived MSCs migrate to the environment from which breast cancer cells originate [37]. Similarly, McLean et al. (2011) reported in their study that they found results demonstrating that MSCs in the microenvironment of ovarian tumors promote tumorigenesis [38].

Cytokines regulate the growth, signaling, and differentiation of both stromal cells and tumor cells. It has been reported that cytokines secreted by stromal cells can influence the behavior of malignant cells, while cytokines produced by cancer cells provide optimal growth conditions within the tumor microenvironment [39]. In our study, we determined the concentrations of IL-8 and IL-6 by ELISA method after co-culturing cancer cells and stromal cells from malignant and healthy breast tissue to determine the concentrations of cytokines thought to originate from stromal cells in the cancer microenvironment.

IL-8, a cytokine of the chemokine family classified as a neutrophil chemoattractant, has been reported to play an important role in tumor progression and metastasis in various human cancers. It is claimed that IL-8 in tumor and tumor microenvironment may contribute to tumor progression through its possible functions in regulating angiogenesis, cancer cell growth and survival, and tumor cell motility [40–42].

As a result of the ELISA assay we performed to determine IL-8 levels, we found that the release of IL-8 increased significantly after co-culturing stromal cells from healthy and malignant breast tissue and cancer cells. We even observed that this increase was higher after co-cultures with malignant stromal cells than with healthy cells. Similarly, studies have found that in addition to the interaction between cancer cells and stromal cells, the expression of IL-8 and other growth factors in stromal cells can also stimulate cancer cells [40–42].

One of the cytokines that play an important role in carcinogenesis is IL-6. It is known that IL-6, a multifunctional cytokine, plays an important role in the biological activities of various cell types, including tumor cells, as well as in controlling growth and differentiation in various malignant states [43–45]. As a result of the ELISA assay we performed to determine the IL-6 level, we found that the level of IL-6 released

into the medium increased significantly after co-culture with stromal cells, and the increase was more pronounced after co-culture with malignant stromal cells. De Luca et al (2012) reported that IL -6 and VEGF secreted by MSCs can promote breast cancer cell migration as paracrine factors [46].

One of the most important features of the cancer cell is the observed changes in gene expression. As a result of these changes, the cells may acquire the ability to proliferate uncontrollably. During cancer development, which is a multistep process, there is an accumulation of mutations in various oncogenes and tumor suppressor genes, and the situation varies depending on the tumor type. Activation of oncogenes and inactivation of tumor suppressor genes cause cells to develop some malignant properties such as uncontrolled proliferation, invasion and metastatic ability [47, 48].

Posttranscriptional gene silencing occurs when the mRNA molecule is destroyed or cannot be translated [49]. miRNAs are known to be involved in cellular processes such as proliferation, differentiation, migration, and apoptosis. However, it is also stated that abnormal miRNA expressions are associated with some diseases, including cancer [35, 50, 51].

With the understanding that miRNAs can be effective in carcinogenesis, the changes in the expression levels of miRNAs in different cancer types compared with normal tissues have been examined and it has been determined that there are differences. With the determination of these differences, miRNAs that are effective in human cancers and their pathological roles have been revealed and continue to be revealed. In addition, determining the expression profiles of miRNAs is also very important for the classification of cancers [5, 13, 14, 17, 35, 50].

As a result of the studies, it was stated that miRNAs function as a new class of oncogene and tumor suppressor genes. miRNAs can acquire oncogenic or tumor-suppressive properties depending on the mRNAs they target. MiRNAs whose task is to control the expression of oncogenes are referred to as tumor suppressor miRNAs. Decreased expression of these miRNAs leads to increased expression of the oncogene and ultimately to tumorigenesis or progression. Contrary to tumor suppressor miRNAs, miRNAs expressed as onco-mir are known to function to increase the development of cancer by decreasing the expression of their target tumor suppressor gene [5, 8, 9, 17, 51].

In our study, after co-cultures of stromal cells and cancer cells obtained from healthy and malignant breast tissues, breast cancer-related let7, miR-17-5p expression levels of genes such as HMGA2, AIB1 belonging to the pathways associated with these miRNAs were determined quantitatively by real-time PCR in all experimental groups.

Similar to our findings, Lee and Dutta revealed that there is a reciprocal relationship between the HMGA2 oncogene and the growth suppressor let-7. In addition, they hypothesized that HMGA2 overexpression during tumorigenesis may occur in conjunction with let-7-dependent pathways [52].

As a result, these results, which are also compatible with the literature, made us think that the cells we isolated from the malignant breast tissue microenvironment had high cancer-related properties, however,

when they were in the same environment with cancer cells, they encouraged the increase of malignant properties of cancer cells.

In our study, we determined that the expression levels of the AIB1 gene, which is known to act as an oncogene, were higher in both cultures of malignant stromal cells alone and co-cultures with cancer cells compared to cultures of cancer cells alone. We found that miR-17-5p expression levels in the opposite direction of the AIB1 gene expression levels were significantly lower after co-cultures of malignant breast tissue-derived stromal cells with cancer cells compared to cultures alone of cancer cells.

Similar to our study, Hossain et al. (2006) reported that mir-17-5p is a potential translational suppressor of AIB1 oncogene in the control of breast cancer cell proliferation and their results revealed that mir-17-5p has a tumor suppressor role in breast cancer cells [53]. Li et al. (2011) also found that mir-17-5p was expressed at high levels in MDA-MB-231 breast cancer cells [54].

Recent studies have shown that the regulatory role of miRNAs in the process of carcinogenesis is not limited to cancer cells, but is also related to tumor stroma activation and its transition to a cancer-associated state [20]. Based on the view that MSCs in the tumor microenvironment support tumor cell development and progression, we can say that our results from our experiments are consistent with the literature. Namely, when we culture stromal cells isolated from the tumor microenvironment of malignant breast tissue and tumor cells in the same medium, we hypothesize that the oscillations of some stromal cell-derived factors increase as a result of their interaction with each other, which supports cancer cell proliferation and tumor progression. Moreover, the changes we observed in some miRNAs and gene expressions that function as oncogenic and tumor suppressors suggest that stromal cells may enter a cancer-associated state and contribute to the increase in malignant properties of cancer cells.

Since the initial identification of miRNAs, many studies have been conducted to understand their formation and mechanism of action. In the studies that have been conducted, it has been shown that disorders in the regulation of miRNAs are involved in the development of many diseases, especially cancer. However, more studies are needed to fully understand their functional significance, define their targets and realize their therapeutic use.

Conclusion

With a better understanding of the properties of cells in the microenvironment known to contribute to the development of cancer, we believe that this is a breakthrough study for the development of new protocols involving the tumor stroma/microenvironment, apart from treatment approaches targeting only cancer cells. Based on our results, we assume that the oscillations of some stromal cell-derived factors increase as a result of their interaction, especially in the co-cultures of malignant breast tissue-derived stromal cells and cancer cells, and that this situation promotes cancer cell proliferation and tumor progression. Moreover, we can say that our study supports the detailed understanding of the cellular and molecular mechanisms of cancer and the effects of tumor microenvironment on tumorigenesis.

As a result, the number of cancer-related deaths is increasing day by day and conventional treatment methods are inadequate. Considering the fact that miRNAs have been shown to play an important role in cancer development in recent studies, the idea that these small molecules can be used in diagnosis and treatment is becoming stronger day by day. In this context, we believe that new treatment approaches involving the tumor microenvironment and using miRNAs as markers are promising.

Declarations

Ethical approval: This study was reviewed and approved by Kocaeli University Clinical Research Ethics Committee. **Code of research protocol:** KAEK 2012/35

Conflict of interests: The authors declare no potential conflicts of interest.

References

1. Arnold CP, Tan R, Zhou B, Yue S-B, Schaffert S, Biggs JR et al (2011) MicroRNA programs in normal and aberrant stem and progenitor cells. Genome Res 21(5):798–810
2. Heneghan H, Miller N, Lowery A, Sweeney K, Kerin M (2009) MicroRNAs as novel biomarkers for breast cancer. Journal of oncology 2010:7
3. Ji Q, Karnak D, Hao P, Wang R, Xu L (2010) No small matter: microRNAs–key regulators of cancer stem cells. Int J Clin Exp Med 3(1):84
4. Pucci S, Mazzarelli P (2011) MicroRNA dysregulation in colon cancer microenvironment interactions: the importance of small things in metastases. Cancer Microenvironment 4(2):155–162
5. Saydam F, Değirmenci İ, Güneş HV (2011) MikroRNA'lar ve kanser. Dicle Tıp Dergisi 38(1):113–120
6. Winter J, Diederichs S (2011) MicroRNA biogenesis and cancer. Methods Mol Biol 676:3–22
7. Michlewski G, Cáceres JF (2019) Post-transcriptional control of miRNA biogenesis. RNA 25:1–16
8. Saliminejad K, Khorram Khorshid HR, Fard SS, Ghaffari SH (2018) An overview of microRNAs: Biology, functions, therapeutics and analysis methods. J Cell Physiol 234:5451–5465
9. Syeda ZA, Saratu Langden SS, Munkhzul C, Lee M, Song SJ (2020) Regulatory Mechanism of MicroRNA Expression in Cancer. İnternational Journal of Molecular Sciences 21:1723
10. Fridrichova I, Zmetakova I (2019) MicroRNAs Contribute to Breast Cancer Invasiveness. Cells 8:1361

11. Toden S, Zumwalt TJ, Goel A (2020) Non-coding RNAs and potential therapeutic targeting in cancer. *BBA-Reviews on Cancer* 1875(2021):188491

12. Chen P-S, Su J-L, Hung M-C (2012) Dysregulation of microRNAs in cancer. J Biomed Sci 19(1):90

13. Çelik Aşçı D, Koşar Aslan P, Özçelik N (2013) MikroRNA'lar ve kanser ile ilişkisi. SDÜ Tıp Fakültesi Dergisi 20(3):121−127

14. Küçükhüseyin Ö, Öztürk O (2013) miRNAlar ve Meme Kanserindeki Etkileri.*Deneysel Tıp Araştırma Enstitüsü Dergisi*3(5)

15. Sassen S, Miska EA, Caldas C (2008) MicroRNA—implications for cancer. Virchows Arch 452(1):1−10

16. Tuğ E, Erol D, Yüce H (2008) MikroRNA'lar ve İnsan Hastalıkları. İzzet Baysal Tıp Fakültesi Dergisi 3(1):24−28

17. Tunalı NE, Tiryakioğlu NO (2010) Kanserde MikroRNA'ların Rolü. Turkiye Klinikleri Journal of Medical Sciences 30(5):1690−1700

18. Mattiske S, Suetani RJ, Neilsen PM, Callen DF (2012) The oncogenic role of miR-155 in breast cancer. Cancer Epidemiology Biomarkers & Prevention 21(8):1236−1243

19. Negrini M, Calin GA (2008) Breast cancer metastasis: a microRNA story. Breast Cancer Res 10(2):203

20. Soon P, Kiaris H (2013) MicroRNAs in the tumour microenvironment: big role for small players. Endocrine-related Cancer 20(5):R257−R267

21. Mehraj U, Dar AH, Wani NA, Mir MA (2021) Tumor microenvironment promotes breast cancer chemoresistance. Cancer Chemother Pharmacol 87:147−158

22. Cuiffo BG, Karnoub AE (2012) Mesenchymal stem cells in tumor development: emerging roles and concepts. Cell Adh Migr 6(3):220−230

23. Esquivel-Chirino C, Esquivel-Soto J, Morales-González JA, Sánchez DM, Ventura-Gallegos JL, Hernández-Mora LE et al (2013) Inflammatory Environmental, Oxidative Stress in Tumoral Progression. *Oxidative Stress and Chronic Degenerative Diseases - A Role for Antioxidants* 187-208

24. Nery AA, Nascimento IC, Glaser T, Bassaneze V, Krieger JE, Ulrich H (2013) Human mesenchymal stem cells: from immunophenotyping by flow cytometry to clinical applications. Cytometry 83(1):48−61

25. Mbeunkui F, Johann DJ Jr (2009) Cancer and the tumor microenvironment: a review of an essential relationship. Cancer Chemother Pharmacol 63(4):571−582

26. Sun Z, Wang S, Zhao RC (2014) The roles of mesenchymal stem cells in tumor inflammatory microenvironment. J Hematol Oncol 7(1):14

27. Yang X, Hou J, Han Z, Wang Y, Hao C, Wei L et al (2013) One cell, multiple roles: contribution of mesenchymal stem cells to tumor development in tumor microenvironment. *Cell Biosci* 3(5)

28. Asadi F, Lazennec G, Jorgensen C (2014) Importance of Stromal Stem Cells in Prostate Carcinogenesis Process.*Cancer Stem Cells Theories and Practice*157−184

29. Polyak K, Kalluri R (2010) The role of the microenvironment in mammary gland development and cancer. Cold Spring Harb Perspect Biol 2(11):a003244

30. Chen D-R, Lu D-Y, Lin H-Y, Yeh W-L (2014) Mesenchymal Stem Cell-Induced Doxorubicin Resistance in Triple Negative Breast Cancer.BioMed research international, 2014:10 pages

31. Lim PK, Bliss SA, Patel SA, Taborga M, Dave MA, Gregory LA et al (2011) Gap Junction–Mediated Import of MicroRNA from Bone Marrow Stromal Cells Can Elicit Cell Cycle Quiescence in Breast Cancer Cells. Cancer Res 71(5):1550–1560

32. Wang Q, Li T, Wu W, Ding G (2020) Interplay between mesenchymal stem cell and tumor and potential application. Hum Cell 33:444–458

33. Zhang L-N, Zhang D-D, Yang L, Gu Y-X, Zuo Q-P, Wang H-Y et al (2020) Roles of cell fusion between mesenchymal stromal/stem cells and malignant cells in tumor growth and metastasis. *The FEBS Journal* 288(2021):1447–1456

34. Kothari C, Diorio C, Durocher F (2020) The Importance of Breast Adipose Tissue in Breast Cancer. Int J Mol Sci 21:5760

35. Zhao L, Sun Y, Hou Y, Peng Q, Wang L, Luo H et al (2012) MiRNA expression analysis of cancer-associated fibroblasts and normal fibroblasts in breast cancer. Int J Biochem Cell Biol 44(11):2051–2059

36. Guan J, Chen J (2013) Mesenchymal stem cells in the tumor microenvironment. Biomedical reports 1(4):517–521

37. Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW et al (2007) Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature 449(7162):557–563

38. McLean K, Gong Y, Choi Y, Deng N, Yang K, Bai S (2011) Human ovarian carcinoma–associated mesenchymal stem cells regulate cancer stem cells and tumorigenesis via altered BMP production. J Clin Investig 121(8):3206–3219

39. Lewis AM, Varghese S, Xu H, Alexander HR (2006) Interleukin-1 and cancer progression: the emerging role of interleukin-1 receptor antagonist as a novel therapeutic agent in cancer treatment.*Journal of translational medicine*4(1)

40. Singh JK, Simões BM, Howell SJ, Farnie G, Clarke RB (2013) Recent advances reveal IL-8 signaling as a potential key to targeting breast cancer stem cells. Breast Cancer Res 15(4):210

41. Waugh DJ, Wilson C (2008) The interleukin-8 pathway in cancer. Clin Cancer Res 14(21):6735–6741

42. Yuan A, Chen J, Yao P-L, Yang P-C (2005) The role of interleukin-8 in cancer cells and microenvironment interaction. Front Biosci 10:853–865

43. Conze D, Weiss L, Regen PS, Bhushan A, Weaver D, Johnson P, Rincón M (2001) Autocrine production of interleukin 6 causes multidrug resistance in breast cancer cells. Cancer Res 61(24):8851–8858

44. Dethlefsen C, Højfeldt G, Hojman P (2013) The role of intratumoral and systemic IL-6 in breast cancer. Breast Cancer Res Treat 138(3):657–664

45. Knüpfer H, Preiß R (2007) Significance of interleukin-6 (IL-6) in breast cancer. Breast Cancer Res Treat 102(2):129–135

46. De Luca A, Lamura L, Gallo M, Maffia V, Normanno N (2012) Mesenchymal stem cell-derived interleukin-6 and vascular endothelial growth factor promote breast cancer cell migration. J Cell Biochem 113(11):3363–3370

47. Aslan G (2010) Tumour Immunology. Turk J Immunol 15(1):7–13

48. Öncel M (2012) Matriks Metalloproteinazlar ve Kanser. Eur J Basic Med Sci 2(3):91–100

49. Bodur E, Demirpençe E (2010) Kodlamayan RNA'lar ve gen susturumu. Hacettepe Tıp Dergisi 41:82–89

50. Frankel LB, Christoffersen NR, Jacobsen A, Lindow M, Krogh A, Lund AH (2008) Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. J Biol Chem 283(2):1026–1033

51. Elizabeth O, Lal A (2010) MicroRNAs and their target gene networks in breast cancer. Breast Cancer Res 12:201:1–10

52. Lee YS, Dutta A (2007) The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. Genes Dev 21(9):1025–1030

53. Hossain A, Kuo MT, Saunders GF (2006) Mir-17-5p regulates breast cancer cell proliferation by inhibiting translation of AIB1 mRNA. Mol Cell Biol 26(21):8191–8201

54. Li H, Bian C, Liao L, Li J, Zhao RC (2011) miR-17-5p promotes human breast cancer cell migration and invasion through suppression of HBP1. Breast Cancer Res Treat 126(3):565–575

Figures
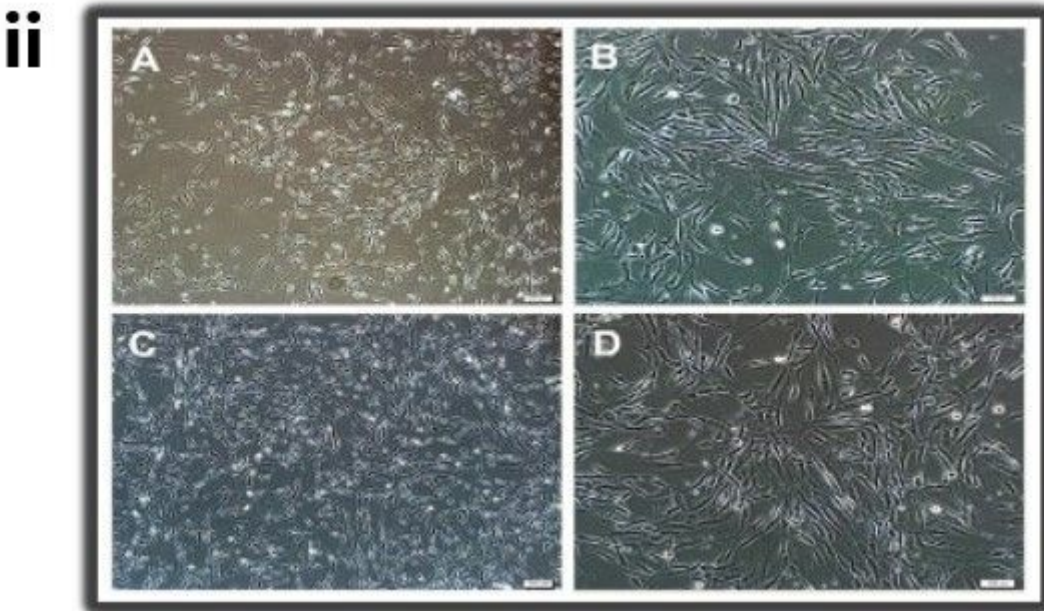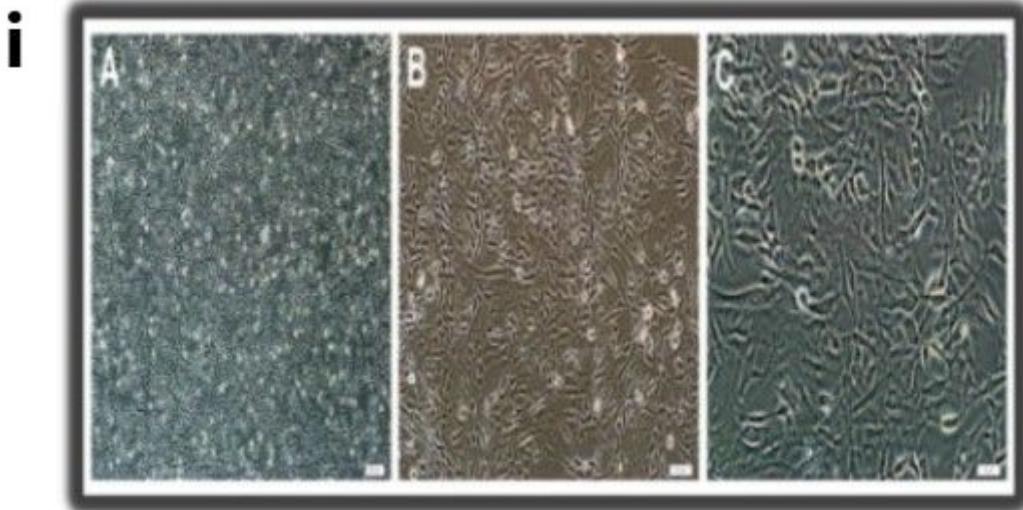
Figure 1

(i)Phase contrast micrographs (A-C) of hHBT-MSCs at P3, day 2 (magnification: A-4X, B-10X, C-20X). (ii) Phase-contrast microscopic views of hMBT-MSCs (A, B) passage 0 – 6th day; (C, D) passage 3 – 2nd day; are observed (Magnification: A-C: 4X, B-D: 10X).
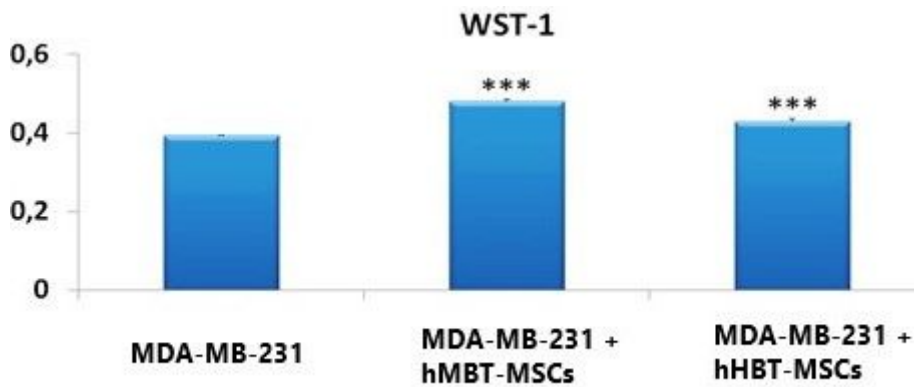
Figure 2

The rate of change of the growth capacity of the experimental groups after 4 days of co-culture with the WST-1 test (***: p≤0.001).
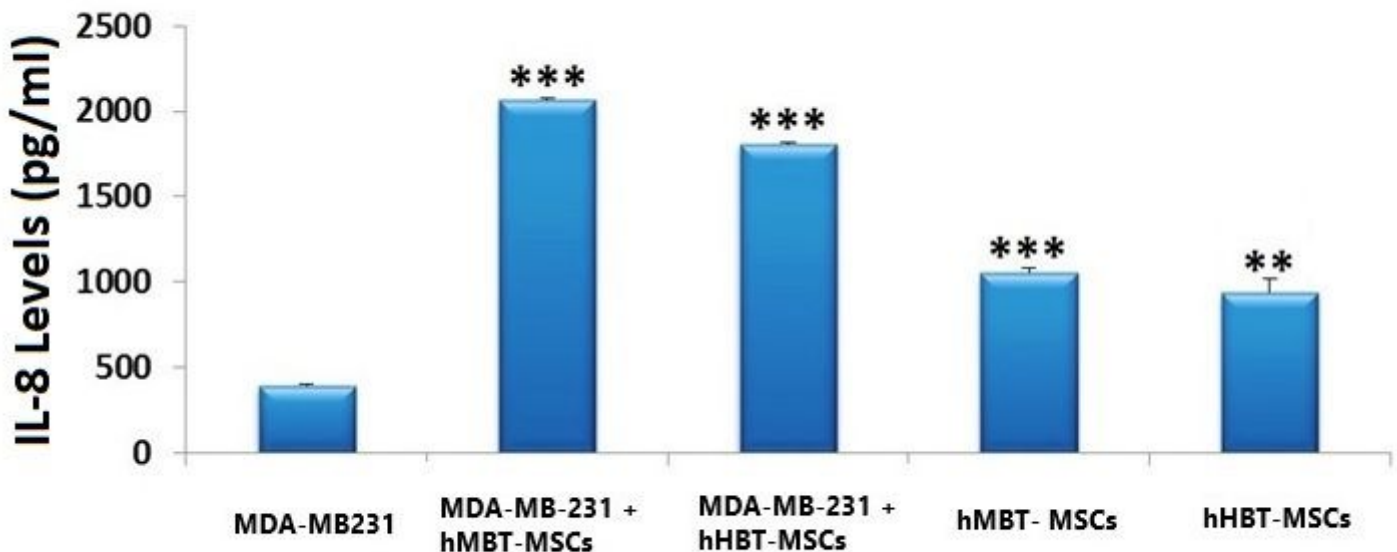

Figure 3

Showing the levels of IL-8 released into the supernatant by ELISA method (***:p≤0.001, **:p≤0.01 Indicates the statistical significance levels determined as a result of the comparison of the experimental groups with respect to the MDA-MB-231 group).
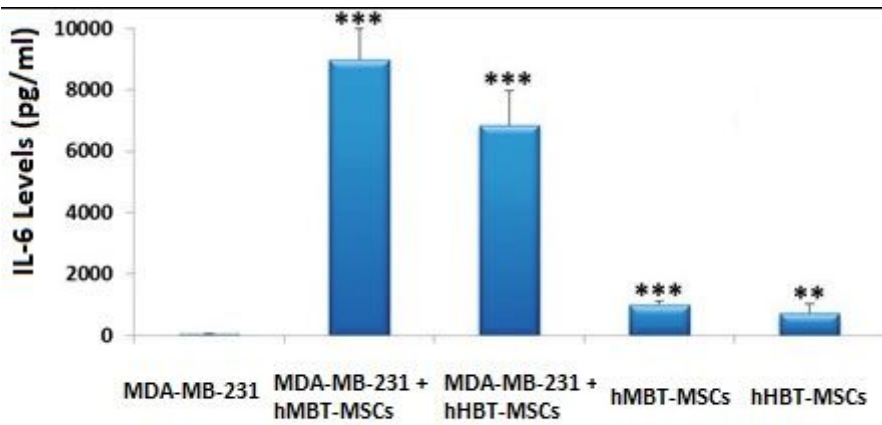
Figure 4

Showing the levels of IL-6 released into the supernatant by ELISA method (***: p≤0.001, **: p≤0.01 refers to the statistical significance levels determined as a result of comparing the experimental groups with the MDA-MB-231 group).
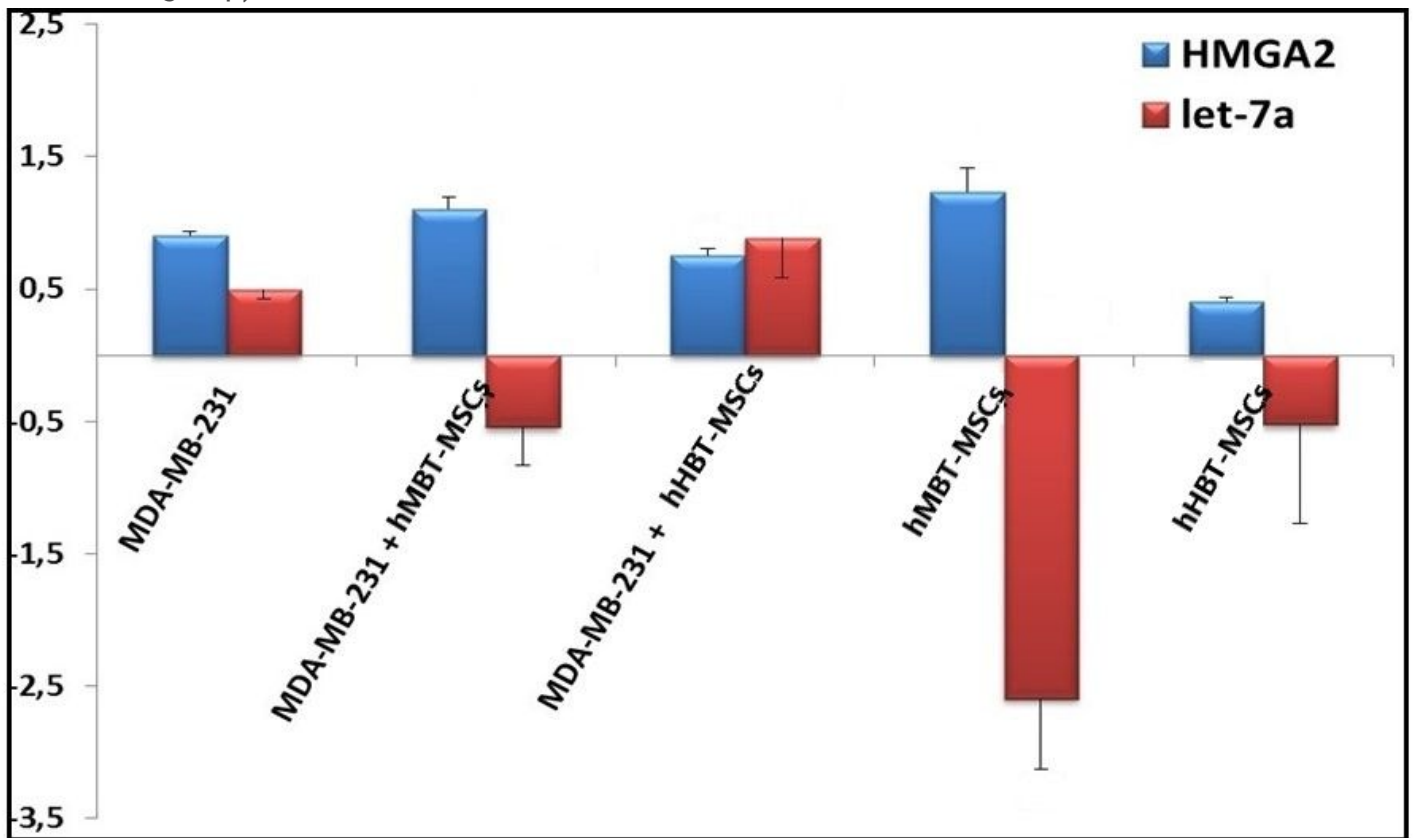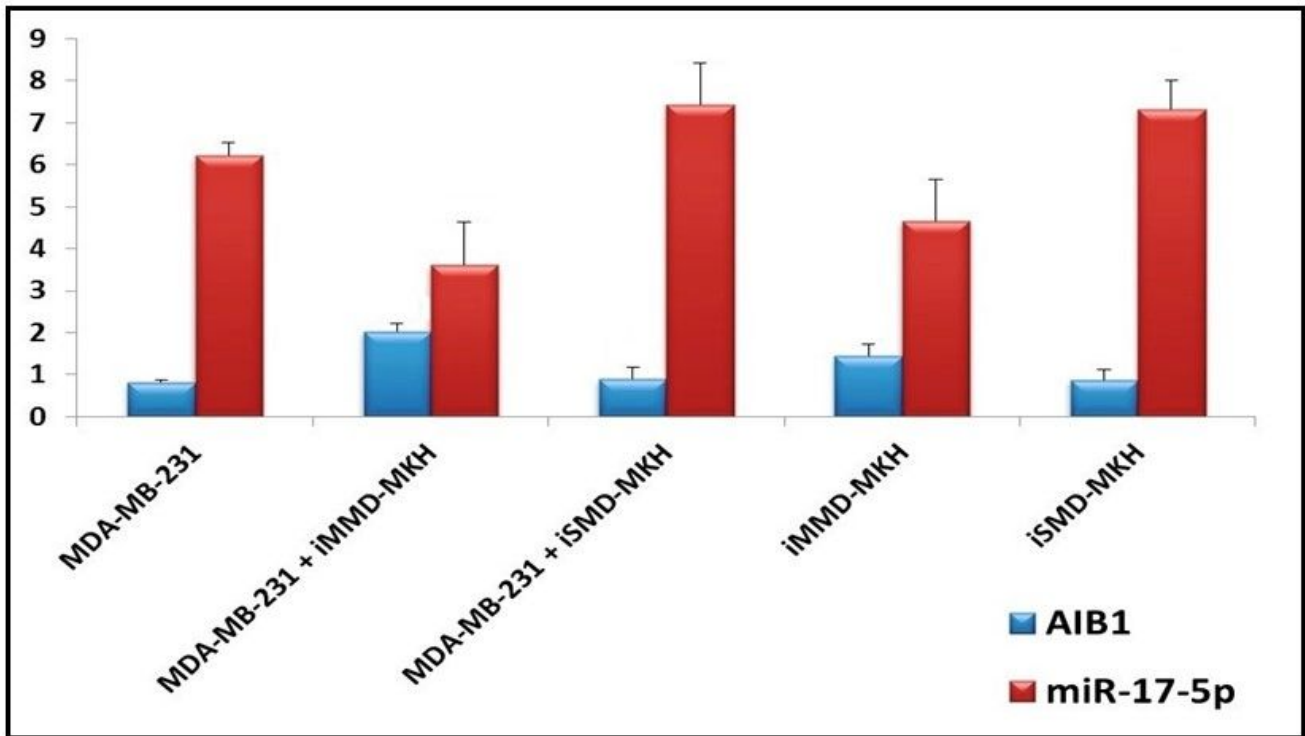

Figure 5

Analysis results of HMGA2 gene and let-7a expression levels by real-time-PCR (X-axis shows experimental groups, Y-axis shows fold changes according to reference gene).

Figure 6

Analysis results of AIB1 gene and miR-17-5p expression levels by real-time-PCR (X-axis shows experimental groups, Y-axis shows fold changes for reference gene).

Supplementary Files

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