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Original Research Article

Determination of Micro RNA 126/126* and Their Target Gene Epidermal Growth Factor Like-7 Expression Profiles in Turkish Breast Cancer Patients

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Abstract

***Micro RNA-126 (miR-126)* and its target gene, *Egfl-7*, are known to enhance apoptotic processes and also play a role in vascular growth through the regulation of vascular endothelial growth factor (VEGF)-mediated signalling, angiogenesis, and vascular integrity. In this study, we aimed to evaluate differences in the expression levels of *miR-126*, its complementary *miR-126** and *Egfl-7* in tumour specimens and peripheral blood mononuclear cells (PBMC) of breast cancer and metastasis. The samples used in this study were provided by the Eskisehir Osmangazi University, Medical Faculty, the Departments of General Surgery and Radiation Oncology. Real Time Polymerase Chain Reaction (RT-PCR) was used to determine the gene expression levels in PBMC and breast cancer tumours. Our study showed that *miR-126 and miR-126** expressions in breast cancer are significantly decreased relative to those in PBMC in healthy controls ($P < 0.001$) and breast cancer tumour specimens ($P \leq 0.001$). Furthermore, a statistically significant increase in *Egfl-7* gene expression was observed in the PBMC of breast cancer and metastasis ($P \leq 0.001$) and in breast cancer tumour specimens ($P \leq 0.001$). The results from the PBMC and tumour samples suggest that *miR-126* and *Egfl-7* may impact the development and metastasis of breast cancer.**

Key Words: Breast Cancer, *Egfl-7*, Metastasis, *microRNA-126, microRNA-126**

Abbreviations

3'-UTR: 3'-Untranslated Region; Ago: Argonaute Protein; BRCA 1/ 2: Breast Cancer 1 and 2; cDNA: Complementary DNA; dsRNA: Double stranded RNA; Egfl-7: Epidermal Growth Factor Like-7; FISH: Fluorescent Based In Situ Hybridization; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HER-2: Human Epidermal Growth Factor Receptor 2; HUVEC: Human Umbilical Vein Endothelial Cells; IHC: Immunohistochemistry; mRNA: Messenger RNA; miR-126: MicroRNA-126; miR-126*: Complementary of miR-126; miRNA: MicroRNA; PBMC: Peripheral Blood Mononuclear Cells; PDCD9: Programmed Cell Death Protein 9; RISC: RNA-Induced Silencing Complex; RT-PCR: Real-Time Polymerase Chain Reaction; sRNA: Small RNA; TIMP9: Human Tissue Inhibitors of Metalloproteinase 9;VEGF: Vascular Endothelial Growth Factor

INTRODUCTION

Small RNAs (sRNA), are double- stranded (dsRNA), 18-29 nucleotide non-coding RNAs. These RNAs are encoded in intergenic regions, which are large areas that form of DNA, and bind the non-transcriptional parts of mRNAs (Saydam et al., 2011; Ventura and Jacks, 2009). Small non-coding RNAs are small RNAs and are involved in epigenetic mechanisms. Most previous studies have shown that epigenetic regulation, especially by micro RNAs (miRNAs; miRs), has an impact on tumour formation (Saito et al., 2006; Yanaihara et al., 2006). miRNAs are small regulatory RNA molecules that play a part in physiological and pathological processes such as apoptosis and stress responses, in addition to proliferation, differentiation, development and regulation of target gene expression (Elbashir et al., 2001; Ventura and Jacks, 2009). Furthermore, miRNAs are formed through a two-stage process involving endonucleases named Drosha and Disher as well as hairpin- shaped precursors. In the biogenesis of miRNA, an uncertainty exists as to which double-stranded miRNA chain will remain following the trimming of the hairpin structure in the precursor. Either of the strands may have a role in the miRNA-induced effects. miRNAs play a role in cellular differentiation and growth using mRNA degradation and translational inhibition mechanisms (Mallick et al., 2010). The most important function of miRNAs is translational inhibition or degradation of mRNA through the alteration of gene activation (Saydam et al., 2011; Ventura and Jacks, 2009). Various miRNAs have been identified to act as tumour suppressors, oncogenes or modulators of cancer cells and metastasis (Cho, 2010). The same miRNA molecule has been shown to exhibit different levels of expression in different cancer types. miRNAs have the potential to be utilized for the diagnosis and therapy of cancers(Huang et al., 2011; Shenouda and Alahari, 2009).Some studies regarding miRNAs both reported the identified miRNA biomarkers and decrypted their target genes and underlying mechanisms (Cho, 2010).

miR-126 and its complementary *miR-126** are molecules that play a part in the formation of breast cancer and metastasis. These molecules have also been shown to have tumour suppressor properties in cellular movements such as migration, invasion, and adhesion in cell lines (Ren and Kang, 2013). *miR-126* and *miR-126** were first discovered in a tissue-specific study in 2002 by Lagos-Quintana et. al (2002). In vertebrates, mature *miR-126* is biologically encoded from the *Egfl-7* gene (Fitch et al., 2004; Parker et al., 2004; Png et al., 2011). *miR-126* is located in intron 7 of the 9th chromosome 9p34.3 region, known as *Egfl-7* (Crawford et al., 2008; Ebrahimi et al., 2014). Furthermore, *miR-126* and *miR-126** are two of the best known miRNAs that are specific to endothelial cells. *miR-126* and its target gene *Egfl-7* are known to enhance apoptotic processes and also play a role in vascular growth through the regulation of VEGF mediated signalling, angiogenesis, and vascular integrity (Patel and Sauter, 2011). Furthermore, *miR-126* participates in tumour growth, endothelial development and metastatic initiation by the inhibition of proangiogenic factors (Feng et al., 2010). When *miR-126 and miR-126** are free in the cytoplasm, they bind to the 3'UTR region of *Egfl-7*, which is known as VE-statin, and inhibit *Egfl-7* by either degradation or translational inhibition of *Egfl-7* mRNA using the endonuclease activity of the Argonaute protein (Ago) in the RISC complex. In this way, *miR-126* and miR-*126** repress target gene expression. Additionally, *Egfl-7* inhibits smooth muscle cell migration by releasing a chemoattractant, which is a peptide specific for endothelial cells (Campagnolo et al., 2005; Soncin et al., 2003).

In this study, we aimed to determine *miR-126*and *miR-126** and *Egfl-7* expression differences between breast cancer and metastasis in PBMC and tumour specimens.

MATERIALS AND METHODS

Sample Characteristics

Tumour specimens were obtained from 17 patients with primary breast cancer without distant metastasis who underwent surgery. Healthy tissue specimens were also obtained from the adjacent normal tissue of the primary breast cancer patients without distant metastasis during mastectomy. The tissue samples were stored in RNA later solution (Sigma, USA) at -80^{0}C until total RNA isolation was performed. The breast cancer blood specimens were obtained from 30 patients with primary breast cancer without distant metastasis who underwent surgery, and metastatic cancer blood specimens were obtained from 30 patients with metastatic cancer which was prior breast cancer who underwent chemotherapy in the Radiation Oncology Department at the Clinical Hospital of Eskisehir Osmangazi University (Eskisehir, Turkey) between 2010 and 2014 (Ethics committee number: PR-11-03-17-09). 30 healthy people blood samples were derived from healthy women with previously undiagnosed cancer diagnosis or treatment. All of the patients provided written informed consent for the use of specimens, and the study was approved by the Institutional Review Board. The blood samples were used to isolate PBMC and then were stored at -80^{0}C until total RNA isolation was completed.

The mean age of the patients with primary breast cancer without distant metastasis at the time of diagnosis was 51 years (range, 27-73). The mean age of patients with metastatic cancer which was prior breast cancer at the time of diagnosis was 50 years (range, 37-88). All of the metastatic cancer specimens derived from patients

Table 1. Forward and reverse sequences of the primers used in RT-PCR

Primer	Forward Sequence	Reverse Sequence
miR-126	3'- GTCCGCTCGTACCGTGAGTAATA-'5	3'-CCAGTCTCAGGGTCCGAGGTATTC-'5
miR-126*	3'-CGCGCTCATTATTACTTTTGGTA–5'	3'-CCAGTCTCAGGGTCCGAGGTATTC-'5
Egfl-7	5'- TGCGACGGACACAGAGCCTGCA-3'	5'-CAAGTATCTCCCTGCCATCCCA-3'
GAPDH	5'-CGAGGGGGGAGCCAAAAGGG-'3	3'-GAAACTGCGACCCCGACCGT-'5

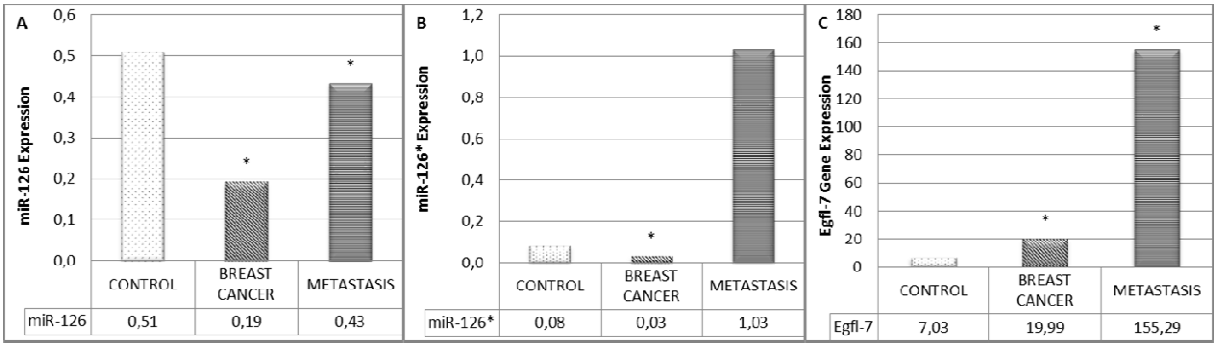

Figure 1. Expression of *miR-126* (A), *miR-126**(B) and *Egfl-7* (C) in PBMC from breast cancer and metastasis (n=30). All of the data were compared to the control group. *P <0.001.

with bone marrow carcinomas which was prior breast cancer. Patients with primary breast cancer without distant metastasis were classified as Grade I (5 cases), Grade II (20 cases) or Grade III (5 cases). Patients with metastatic cancer were classified as Grade I (1 case), Grade II (18 cases) or Grade III (11 cases). Information on the pathological status was collected from the original surgical pathological reports, and the records of surgery and in-patient medical records were also reviewed.

PBMC and Total RNA Isolation

PBMC were isolated from blood samples by the using Fiquol-Histopaque Method (Fiquol PLUS, Amersham, Sweden). Total RNA was isolated from PBMC using the Paris kit (Ambion, USA) and from frozen tissue samples the using Tissue RNA PrepMate kit (Bioneer, Republic of Korea).

RT-PCR

The RNA isolated from PBMC and frozen tissue samples was converted to cDNA through reverse transcription (Bioneer, USA). Expression of *miR–126/126** and *Egfl–7* (Alpha DNA, Montreal, Quebec) was determined with RT-PCR (Strategene MxPro3000, UK). *GAPDH* (Alpha DNA, Montreal, Quebec) was used as the internal control. The forward and reverse primer sequences used in RT-PCR are shown in Table 1.

Statistical Evaluation

The expressions of *miR-126* and *miR-126** and *Egfl-7* was evaluated statistically using one-way ANOVA for variation analyses. The comparisons between the groups of non-normally distributed variables were evaluated by Kruskal-Wallis test and Mann-Whitney multiple comparison tests.

RESULTS

According to the data obtained from PBMC in breast cancer patients, *miR-126* expression [0.19 (0.009-0.785)] decreased compared with negative controls [0.51 (0.04-1.347)]. Additionally, *miR-126** expressions [0.03 (0.006-0.112)] decreased compared with the healthy controls [0.08 (0.034-1.02)] (P ≤ 0.05). *Egfl-7* gene expression [19.99 (2.549-134.219)] increased compared with negative controls [7.03 (1.537-49.874)] (P ≤ 0.001; Figure 1).

The *miR-126* expression data from PBMC in the metastatic cancer which was prior breast cancer showed a decrease [0.43 (0.281-4.199)] compared with negative controls [0.51 (0.04-1.347)] (P ≤ 0.001). *miR-126** expression [1.03 (0.551-8.225)] was increased compared with the healthy controls [0.08 (0.034-1.02)] (P ≤ 0.05). Furthermore, *Egfl-7* gene expression [155.29 (11.632-438.911)] was increased to a statistically significant level compared with the healthy controls [7.03 (1.537-49.874)] (P ≤ 0.001; Figure 1).

In breast cancer tissue specimens, *miR-126* express-

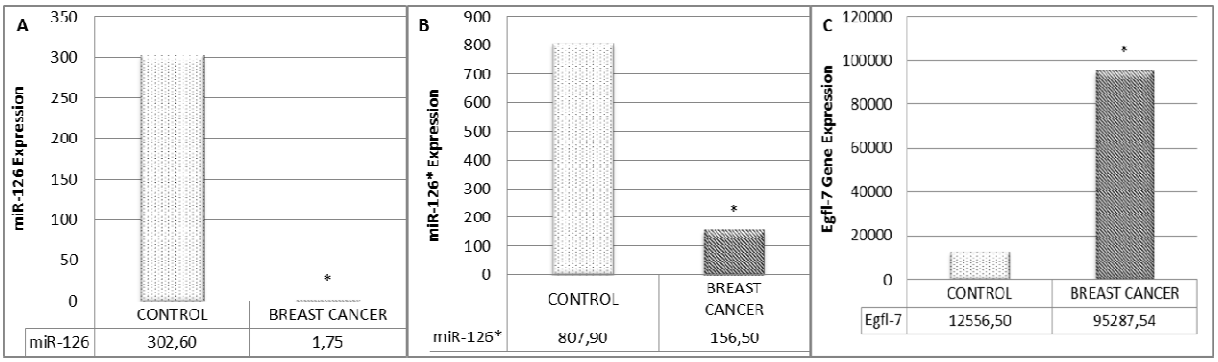

Figure 2. Expression of *miR-126* (A), *miR-126**(B) and *Egfl-7* (C) in breast cancer tumours (n=17).All of the data were compared to the control group. *P <0.001.

ion [1.75 (0.515-16.485)] was decreased compared with the healthy controls [302.6 (36.25-302.62)] (P < 0.001). *miR-126** expression [156.5 (22.24-275.61)] was decreased compared with the healthy controls [807.9 (27.86-807.93)] (P < 0.001). *Egfl-7* gene expression [95287.54 (11636.82-451720.07)] was increased compared with the healthy controls [12556.5 (10369.08-349594.56)] (P < 0.001; Figure 2).

DISCUSSION

Non-coding RNAs, one of the regulators of epigenetic mechanisms, can influence the development of cancer. Study of the mRNA and miRNA expressions, although there were no detected differences in the mRNA expression between normal and cancerous samples, differences in miRNA expression were discovered. Thus, the miRNA expression profiles can be used effectively in confirming the diagnosis and classification of cancer (Fire et al., 1998; Kosaka et al., 2010; Lee et al., 1993; Vimalraj et al., 2013). *miR-126 and miR-126** are biologically encoded from the *Egfl-7* gene in vertebrates (Fitch et al., 2004; Parker et al., 2004). According to various studies, *miR-126*and *miR-126** are largely found in non-cancerous tissues including the heart (Liu et al., 2009), liver(Lagos-Quintana et al., 2002; Landgraf et al., 2007), lung(Izzotti et al., 2009; Landgraf et al., 2007)or human umbilical vein endothelium (HUVEC) (Meister and Schmidt, 2010; Musiyenko et al., 2008). In cancer studies, these results appear to be the opposite. In previously studies, *miR-126* expression was high in healthy control tissues compared to lung (Cho et al., 2009; Yanaihara et al., 2006), stomach (Feng et al., 2010), cervix (Wang et al., 2008), bladder and prostate (Saito et al., 2006) tumours. . In a study on lung tumours, a reduction in miR-126 was demonstrated(Lagos-Quintana et al., 2002). In endothelial and immune cells, miR-126 and miR-155 expression was observed (Zhu et al., 2011). In a mouse xenograft model study to inhibit the metastasis of breast cancer to the lung, *miR-126*and *miR-126** were shown to first inhibit mesenchymal stem cells and then inhibit inflammatory monocytes in the tumour stromal independently (Zhang et al., 2013). Furthermore, in a study on *miR-126* expression, transfecting the oncogene *v-Src* into Cx43KO mouse embryonic brain cell lines resulted in a decrease in *miR-126*and *miR-126** expressions (Li et al., 2009). Liu S. et al. (2012) suggested that the decrease in *miR-126* expression at the inner layers of the uterine gland and supporting tissues caused an increase in the CRK gene and protein expression, an important signalling adaptor protein, during endometriosis (Liu et al., 2012).In breast, colorectal, lung, pancreas and prostate cancers, miR-126 and miR-34a showed low expression by using immunohistochemistry (IHC) and fluorescent- based in situ hybridization (FISH) methods(Sempere et al., 2010).

In a previous study, *miR-210, miR-21, miR-29a* and *miR-126* expressions were decreased in the plasma samples of breast cancer patients who were treated with tratuzumab, an chemoattractant drug that affects epidermal growth factor receptor 2 (HER-2), and the results showed that these miRNAs might be tumour suppressors in breast cancer (Jung et al., 2012). In another study that was performed using sera and tissue samples from patients with breast tumours, *miR-126*, *miR-199a* and *miR-335* showed low expression levels while *miR-21, miR-106a* and *miR-155* showed high expression levels in breast cancer according to tumour type (malignancy and tumour grade), oestrogen and progesterone levels (Wang et al., 2010). In a study by Hoppe R. et al (2013), an increased expression of *miR-126* and *miR-10a* was observed in the prolonged, relapse-free time of primary oestrogen receptor-positive breast cancer following tamoxifen treatment (Hoppe et al., 2013).

Generally, *miR-126* and *miR-126** interact with *Egfl-7* because of sequence similarity (Fish et al., 2008; Meister and Schmidt, 2010; Soncin et al., 2003). A decrease in miR-126* expression in colon (Guo et al., 2008), lung

(Yanaihara et al., 2006) and prostate (Musiyenko et al., 2008) cancers was determined. Thus, the expressions of *miR-126* and miR126* were thought to be similar in all situations. In some cases, the miRNA level is considerably different; however, a study regarding the interaction between *miR-126*, *miR-335* and the risk of breast cancer in patients with a *BRCA 1 / 2* gene mutation and family history of breast cancer, *miR-126* and *miR-335* expression in cancer metastasis was reported to increase (Yang et al., 2011). According to our data, *miR-126** expression decreased only in the PBMC of breast cancer patients. In tumour specimens, *miR-126** expression decreased compared with the healthy controls. The postulated reason for this expression difference between *miR-126* is that any one of the two miRNAs can be involved in the mechanism of action. Also, these miRNAs have different nucleotide sequences and their potential different gene targets. Therefore, miRNAs and their complementary need to be considered as two different miRNAs.In our study, these complementary two miRNAs did not provide the same results in the different samples.

In a study by Ding H. et al. (2014), *miR-126*, *miR-96* and *miR-144* expressions significantly decreased in metastatic cervix cancer compared with non-metastatic cervix cancer (Ding et al., 2014). Some miRNAs decreased in both the benign and malignant breast cancer samples compared with the healthy controls. For example, a study by Tahiri A. et al. suggested that (2014) *miR-126*, *miR-193b* and *miR-193a-3p* expressions decreased in both benign and malignant breast cancer tumours (Tahiri et al., 2014). Research on breast tumours indicated that *miR-126* expression was lower in metastatic breast tumour specimens (Tavazoie et al., 2008). In a study on miRNAs that are related to an anti-angiogenic*TIMP9*, which controls the catalytic activities of matrix metalloproteinase in breast tumours, and *PDCD9*, an apoptotic gene that alters cancer initiation and metastasis (*miR-17-p, miR-126, miR-335* and *miR-30b*), the miRNA expression levels decreased in the breast tumours compared with the healthy controls. However, there were no statistically significant differences between the lymph node metastasis and healthy controls (Hafez et al., 2012). Furthermore, in a different study, the *miR-126*, *miR-127-3p, miR-143, miR-145* and *miR-199a-3p* expression levels decreased in malignant myoepithelioma compared with breast cancer luminal A and, B and basal subtypes in a study on different miRNA expression levels in breast cancer subtypes (Bockmeyer et al., 2011).

Similar to these studies, in our study, the *miR-126* expression level decreased in PBMC and metastasis of breast cancer patients. Complementary sequences showed parallel results in PBMC of breast cancer patients. Additionally, the tumour specimens showed decreased *miR-126* expression compared with the healthy controls.

The *Egfl-7* gene is highly expressed in malignant, invasive cells. Methylation of the *Egfl-7* gene promoter is thought to down regulate*miR-126*and *miR-126** expressions (Zhang et al., 2013). In another study, the absence of *miR-126** due to the transfection of a synthetic anti-miR-126* into LNCaP prostate cancer cells was shown to result in increased PROSTEIN target protein translation, including that of EGFL-7 (Musiyenko et al., 2008). In a study involving non-small cell lung cancer (NSCLC) cell lines, a significant reduction in CRK protein in cells transfected with pre-miR-126 was observed (Crawford et al., 2008). In another study involving NSCLC cell lines, the suppression of *miR-126* with anti-miR-126 was associated with increased *Egfl-7* and its products (Sun et al., 2010). According to our data, *Egfl-7* gene expression was increased in both the tumour specimens and PBMC of patients who had breast cancer and metastasis.

CONCLUSION

Cancer is a complex disease that is associated with medical (pharmacology, genetic, molecular biology etc.) and surgical sciences. Metastasis is the most fatal cancer stage. Therefore, we must first aim to block cancer metastasis. Revealing the condition of the cells in primary cancer and metastasis is an important step towards determining future treatment strategies. According to our data, a low expression of *miR-126* found and*Egfl-7* expression was high in frozen tissue specimens and PBMC of patients with breast cancer and its metastasis. We suggest that both *miR-126*expression decrease and *Egfl-7*expression increase in breast cancer and its metastasis.*miR-126* and *Egfl-7* might be descriptive regions of breast cancer.

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- Eskisehir Osmangazi University, Committee of Human Ethics decided there was no drawback to collecting blood and tissue samples from the patients for this study (Ethics committee number: PR-11-03-17-09).

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