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VEGF and KRAS are Potential Targets of miR-206 Modulation in Triple Negative Breast Cancer

Shaymaa E. El Feky[1,*], Fawziya A.R. Ibrahim[2], Adel Nassar[3], Nadia A. Abd El Moneim[4], Samia A. Ebeid[2], Mohammad A. Ahmad[5], Sanaa Shawky[6], Mohammad M. Nasef[3]

[1]Radiation Sciences Department, Medical Research Institute, Alexandria University, Alexandria, Egypt
[2]Applied Medical Chemistry Department, Medical Research Institute, Alexandria University, Alexandria, Egypt
[3]Chemistry Department, Faculty of Science, Menoufia University, Shebin El-Koam, Egypt
[4]Cancer Management and Research Department, Medical Research Institute, University of Alexandria, Egypt
[5]Military Medical Academy, Alexandria, Egypt
[6]Pathology Department, Medical Research Institute, University of Alexandria, Egypt
*Corresponding author: shaymaa.elfeky@alexu.edu.eg

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Abstract Triple negative is a subtype of breast cancer characterized by lack of expression of hormone receptors (ER, PR and Her2/neu). Due to the limited treatment options, the search for novel treatment targets continues. The aim of this study was to assess the differential expression of miR-206, VEGF and KRAS in TNBC and non-TNBC tissues and cell lines and to evaluate the modulatory effect of miR-206 on the key oncogenic targets VEGF and KRAS. The expression of miR-206, VEGF and KRAS was quantified using real time PCR in both paraffin embedded breast cancer and adjacent tissues as well as in MDA-MB-231 and MCF-7 cell lines. Cell lines were transfected with different concentrations of miR-206 mimic and their viability were assessed using MTT assay. Our results indicated that miR-206 was significantly downregulated in cancerous compared to non-cancerous tissues with a more pronounced downregulation in TNBC than non-TNBC tissues. VEGF and KRAS were significantly upregulated in TNBC compared to non-TNBC and their expression was negatively correlated to miR-206 expression. Transfection of TNBC and non-TNBC cell lines with miR-206 mimic resulted in a dose dependent reduction in cell viability as well as a significant reduction in VEGF and KRAS expression. In conclusion, based on our combined human tissues and cell line-based investigations we can suggest that VEGF and KRAS may be potential targets for miR-206-mediated regulation and that their targeting by miR-206 can be a highly efficient therapeutic strategy in TNBC.

Keywords: *triple negative breast cancer, miR-206, VEGF, KRAS, epigenetic regulation*

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1. Introduction

Triple negative breast cancer (TNBC) is the most aggressive subtype and represent up to 20% of breast malignancy [1]. TNBC is uniquely characterized by lack of expression of surface receptors for estrogen, progesterone and Her2/neu, therefore it doesn't respond to ordinary treatments such as tamoxifen or aromatase inhibitors or therapies that target and inhibit HER2 receptors, such as trastuzumab [2]. This consequently limits its treatment options to cytotoxic agents like cisplatin and doxorubicin despite of their off-target toxicities [3]. The search for molecular therapeutic targets and new potential drugs is therefore mandatory.

MiRNAs are a class of promising emerging regulatory molecules. They are short, non-coding form of RNAs that are differentially expressed in cancer tissues [4]. miRNAs can either act as tumor suppressors or promotors by regulating their target proteins through suppression of gene translation, induction of mRNA degradation or interfering with mRNA post-translational modifications [5,6]. miRNAs are characterized by their ability to modulate several targets with a single hit leading to formation of a network of interacting regulatory molecules. Several reports have linked the dysregulation of miRNA expression with disease development including induction of invasion, metastasis, apoptosis and angiogenesis [7,8]. Furthermore, several miRNAs have been linked to aggressive tumor features in TNBC [9].

MiR-206 has been reported to be down-regulated in several types of cancer including gastric [10], liver [11], lung [12] and breast cancer [13]. Latest reports have linked miR-206 in inhibition of cell proliferation, migration, stemness and metastasis in breast cancer

[14,15]. It has also been associated with advanced clinical stage, and low overall survival [15]. Despite of this extensive research, few studies have focused on the role of miR-206 in TNBC and its potential regulatory targets.

Vascular endothelial growth factor (VEGF) and the Kirsten rat sarcoma viral oncogene homolog (KRAS) are two of the deriving genes in cancer progression [17,18]. Nevertheless, investigations of their regulation in TNBC and the possible connection between the two genes is still limited. Therefore, in the current study we aimed to investigate the possible differential expression of miR-206, VEGF and KRAS in TNBC compared to non-TNBC and normal tissues. We also investigated the regulatory role of miR-206 on genetic expression of VEGF and KRAS in TNBC.

2. Materials and Methods

2.1. Human Breast Cancer Tissue Samples and Patients' Characteristics

Forty-two formalin-fixed, paraffin-embedded (FFPE) archived breast cancer tissues and corresponding apparently normal adjacent breast tissues were collected from BC patients who underwent surgical resection. Samples were deidentified according to the ethical and legal standards then subdivided according to their receptor status into triple negative (n=15) and non-triple negative (n=27) groups. The age of patients ranged from 32-69 years and their clinicopathological characteristics are summarized in Table 1.

Table 1. Clinical Characteristics of Breast Cancer Patients

Characteristic	No.	%
Receptor Status		
Triple Negative	15	35.7%
Non-triple Negative	27	64.3%
Vascular Invasion		
Negative	1	2.3%
Positive	41	97.7%
Lymph Node Involvement		
T0	8	19.0%
T1	9	21.4%
T2	14	33.3%
T3	11	26.3%
Histological Grade		
II	35	83.3%
III	7	16.7%

2.2. Cell Lines and Culture Conditions

The human breast adenocarcinoma triple negative cell line MDA-MB-231 (ATCC$^{(R)}$ HTB-26) and the ER expressing MCF-7 cell line (ATCC$^{(R)}$ HTB-22TM) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 mg/ml of streptomycin sulfate. Cells were allowed to grow in humidified atmosphere at 37°C under 5% CO_2 in 6 and 96-well tissue culture plates.

2.3. Transfection of MDA-MB-231 and MCF-7 Cell Lines with miR-206 Mimic

The miR-206 miScript miRNA mimic (Qiagen Group, USA) was transfected into MDA-MB-231 and MCF-7 cells at using stock concentration of 100 nM. Reverse transfection started with adding miR-206 mimic to 96-well plate followed by the addition of HiPerFect transfection reagent (Qiagen Group, USA) diluted in culture medium. The mixture was Incubated for 10 mins at room temperature to allow formation of transfection complexes. $2x10^4$ cells in culture medium were seeded into each well, on top of the miRNA mimic-HiPerFect Reagent transfection complexes and the plates were incubated at 37 °C with 5% CO_2 for 48 hours. To assess the transfection efficiency, cells were transfected using FAM-labeled miRNA and examined by fluorescent microscope 48 hours following transfection. Evaluation of transfection efficiency by imageJ software showed that the density of emitted fluorescence was correlated to the number of cells and transfection was performed with high efficiency.

2.4. Cell Viability

The viability of Cells MDA-MB-231 and MCF-7 cells were evaluated using the MTT assay. Cells were seeded into 96-well plates. 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma-Aldrich, MO, USA) was added 48h following the treatment. Cells were then incubated for 3 hours. Viable cells are detected by its mitochondrial conversion of the tetrazolium salt MTT into formazan crystals, which can be solubilized for homogenous measurement at OD 540. The control cell viability was defined as 100%.

2.5. Quantitative Determination of miR-206, VEGF and KRAS in FFPE Samples and Cell Lines

Total RNA was extracted from formalin-fixed, paraffin-embedded tissues using RNeasy FFPE Kit (Qiagen Group, USA) and from cell lines using RNeasy Mini Kit (Qiagen Group, USA) according to manufacturer instructions. The purity and concentration of extracted RNA were evaluated by NanoDrop$^{(R)}$ ND-1000 UV-Visible Spectrophotometer (Thermo Fischer Scientific, USA). Total RNA was reverse transcribed using TaqMan™ advanced miRNA cDNA synthesis Kit (Applied Biosystems, USA) for miR-206 and high-capacity cDNA reverse transcription Kit with RNase Inhibitor (Applied Biosystems, USA) for VEGF and KRAS, according to the manufacturer's instructions. Real-time PCR was performed by TaqMan kits (TaqMan® Small RNA assay and Gene expression assay). U6 small nuclear RNA and GAPDH were used as internal controls to normalize the expression of miR-206, VEGF and KRAS respectively. Results were presented as average fold change of target gene in test to control group using $2^{-\Delta\Delta CT}$ formula.

2.6. Statistical Analysis

Data were analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). Quantitative data were described using median and range. Significance of the obtained results was judged at the 5% level. H-test (Kruskal Wallis) was used for abnormally distributed quantitative variables, to compare between more than two groups, and Mann-Whitney test for pairwise comparisons. Correlations between miR-206 and VEGF and KRAS expression was done using Spearman correlation test.

3. Results

3.1. Downregulation of miR-206 and Upregulation of VEGF and KRAS in TNBC Tissues

Quantitative real time PCR results show that miR-206 expression in TNBC tissue is significantly lower than non-TNBC and normal breast tissues (p=0.002 and <0.001 respectively). Expression of miR-206 in non-TNBC tissue was also lower than normal tissues (p=0.042), however, the levels was still higher than the TNBC tissues (Figure 1a). Additionally, the gene expression levels of VEGF and KRAS in TNBC tissues showed a significant elevation compared to normal tissues (p=<0.001 and 0.003 respectively) and to non-TNBC tissues (p= <0.001 and 0.002 respectively). It worth notion that the level of VEGF and KRAS were elevated in non-TNBC compared to normal tissues as well (p=0.003 and 0.030) but were still lower than that in TNBC (Figure 1 b & Figure 1c). The levels of both VEGF and KRAS were inversely correlated to miR-206 expression (Figure 2 a & Figure 2b).

Furthermore, association with clinicopathological data revealed that TNBC patients had significantly higher histological grade (U=39, p=0.003) and increased number of positive lymph nodes involvement (H=10.37, p=0.016). Worse histological grade was also associated with lower levels of miR-206 (U=59, p=0.031) and higher KRAS (U=30.5, p=0.004) expression levels while VEGF did not show a significant difference with tumor grade (U=95.5, p=0.323). None of the studied markers showed a significant association with lymph nodes involvement.

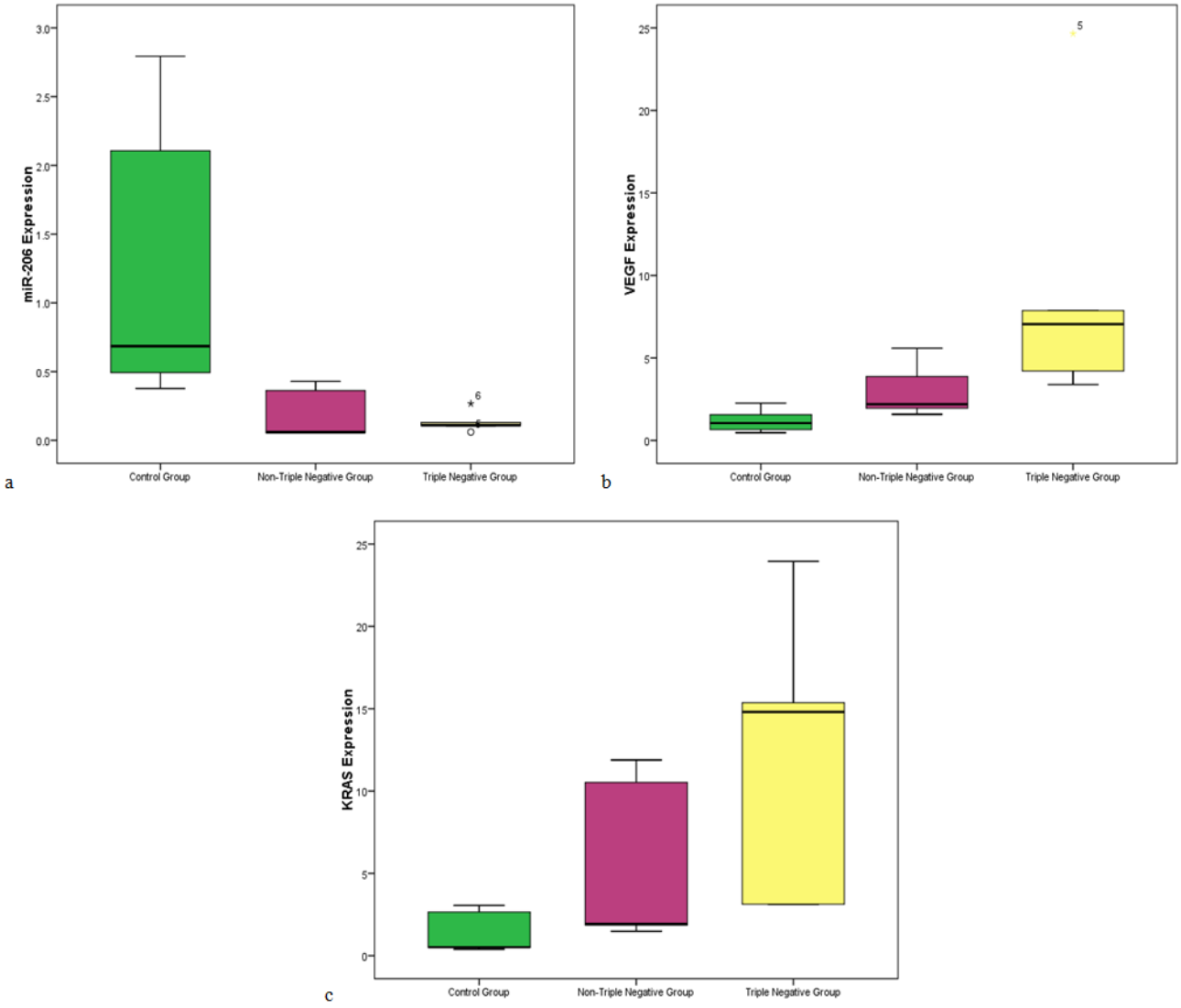

Figure 1. The expression of (a) miR-206, (b) VEGF and (c) KRAS in paraffin embedded (TNBC, non-TNBC and non-cancerous) tissues

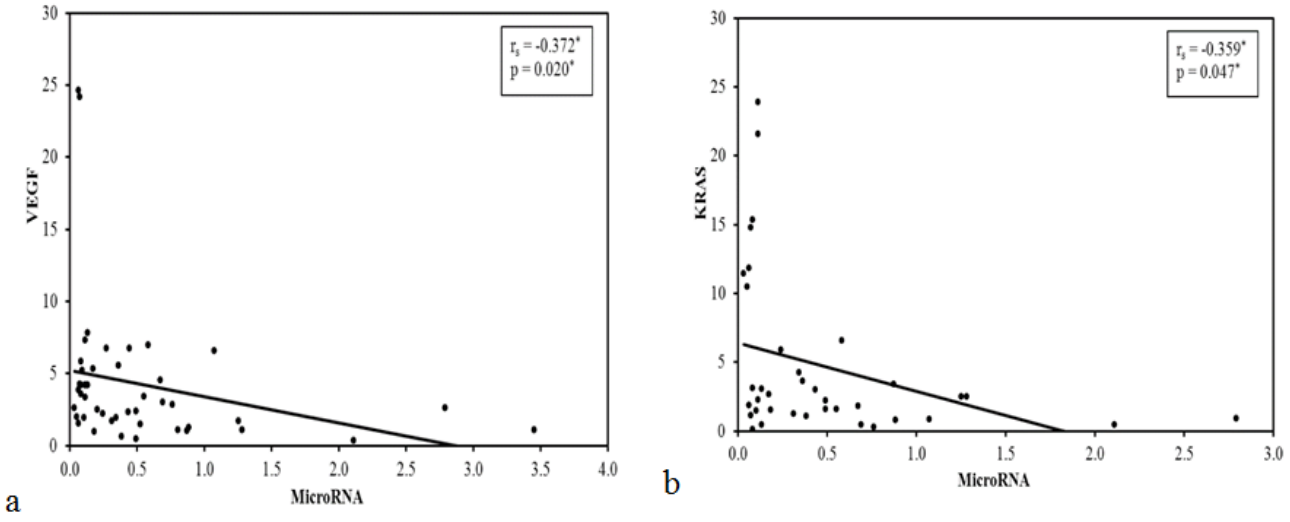

Figure 2. Correlations between miR-206 expression and (a) VEGF and (b) KRAS in breast cancer tissues

3.2. Transfection of MDA-MB-231 and MCF-7 Cell Lines with miR-206 Decreases Cell Viability

To evaluate the effect of miR-206 mimic on MDA-MB-231 and MCF-7 cells viability, cultures were treated with different concentrations of mimic (20, 40, 60, 80 and 100 nM). The viability of cells was assessed using MTT assay and expressed as percent from control at 48 hours post transfection. In MCF-7 cell line, the decrease in viability was dose dependent and started to show a significant difference from control group at miR-206 mimic concentrations of 60, 80 and 100 nM (p=0.036, 0.016 and 0.016 respectively) (Figure 3a). MDA-MB-231 viability showed a significant elevation compared to control group at miR-206 mimic concentration of 20 nM. Nevertheless, with increasing the mimic concentration, the viability decreased in a dose-dependent manner and was significantly different than the untreated group at mimic concentration of 60 nM and more (p=0.029) (Figure 3b).

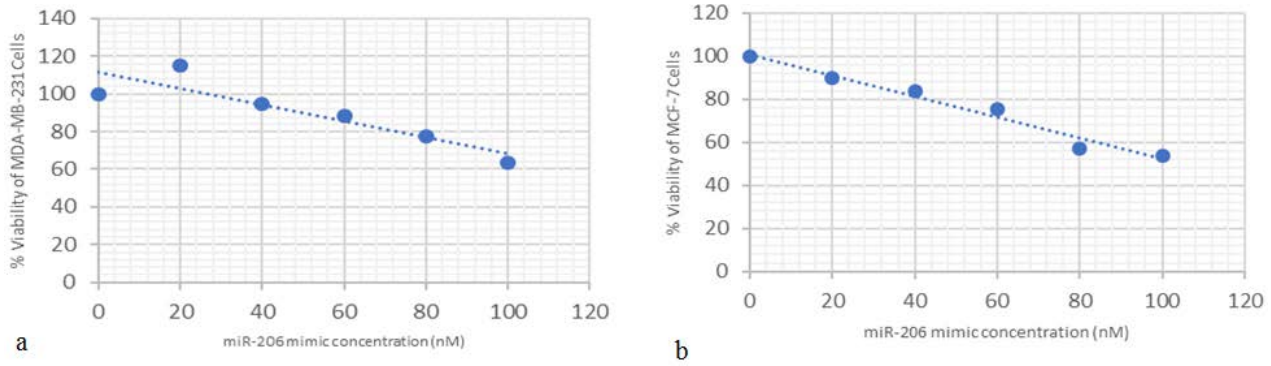

Figure 3. Viability of (a) MDA-MB-231 and (b) MCF-7 cell lines after transfection with (0, 20, 40, 60, 80, and 100 nM of miR-206 mimic)

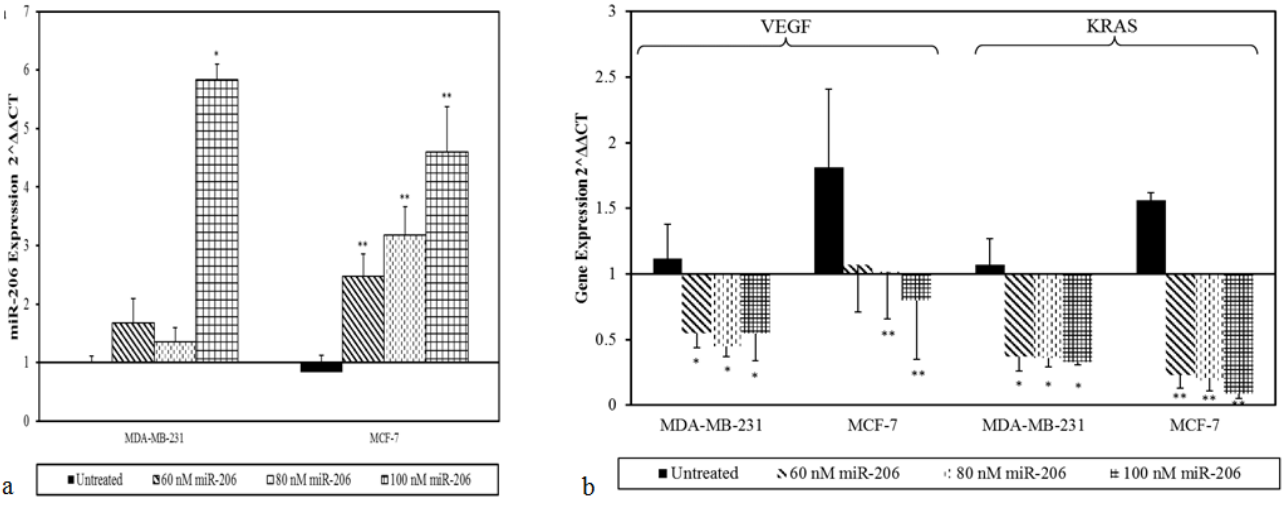

Figure 4. The Quantitative Determination of (a) miR-206, (b) VEGF and KRAS expression in MDA-MB-231 and MCF-7 cell lines with and without transfection with miR-206 mimic (where * significantly different from MDA-MB-231 control, ** significantly different from MCF07 control at p≤0.05)

3.3. VEGF and KRAS Expression is Regulated by miR-206 in TNBC and Non-TNBC Cell Lines

Quantitative determination of miR-206 expression was done with and without transfection with 60, 80 and 100 nM miR-206 mimic. Our results indicated that there was no significant difference between the basal levels of miR-206 in both MDA-MB-231 and MCF-7 cell lines (p=0.476). Figure 4a represents that miR-206 levels were significantly elevated at all treatment concentrations compared to nontreated cells in non-triple negative MCF-7 cell line (p=0.029, 0.043 and 0.016 respectively). On the other hand, transfection with miR-206 lead to a significant elevation in MDA-MD-231 miR-206 levels only at 100 nm (p=0.046).

The elevation in miR-206 expression was associated with a corresponding down-regulation of both VEGF and KRAS genes in both cell lines. For VEGF, the expression was significantly decreased in MCF-7 cells treated with 80 and 100 nM of miR-206 mimic (p=0.036, 0.036). MDA-MB-321 cells also showed a significant reduction in VEFG expression at all treatment concentrations (p=0.026, 0.015 and 0.026 respectively). Regarding KRAS expression, both cell lines showed a reduction in expression associated with miR-206 mimic treatment in a dose-dependent manner with p values (0.03, 0.05 and 0.024) for MCF-7 and (0.002, 0.002 and 0.004) for MDA-MB-231 (Figure 4b).

4. Discussion

The aberrant expression of miRNAs is notable in many types of cancer owing to their crucial role in epigenetic regulation [19]. Several miRNAs promote the tumorigenesis through the control of processes like tumor invasion, metastasis, apoptosis and angiogenesis [20]. The present work revealed the expression of miR-206 was significantly downregulated both in TNBC and non-TNBC tissues with a predominant downregulation in the triple negative subgroup. MiR-206 down-regulation was also found to be associated with higher histological grade which was more prevalent in TNBC patients. Previous reports have supported the role of miR-206 in the progression of many types of cancers [21]. Low levels of miR-206 expression have been correlated with tumor size and advanced pathological stage which suggested its use as a promising prognostic marker in breast cancer [22,23].

The fact that the expression of miR-206 was significantly down-regulated in TNBC compared to non-TNBC strongly suggested its involvement in the development of more aggressive characteristics associated with TNBC subtype. These findings are in accordance with results of Salgadoa et al who also reported that TNBC tissues and cell lines expressed miR-206 less than non-TNBC equivalents [23]. Downregulation of miR-206 in TNBC have been linked with repression of cell migration via direct targeting of many proteins including actin-binding protein coronin 1C [24].

Given the potential role of miR-206 in TNBC progression, the mechanisms by which miR-206 exerts its function drew our attention. In this study, we correlated the levels of miR-206 expression with KRAS and VEGF which represent key players in essential activated pathways in cancer progression. VEGF serves a key functional gene in the process of angiogenesis, while KRAS is a remarkable deriving gene in breast cancer. The interplay between the two genes and their regulation by miR-206 is an interesting approach in understanding their role in TNBC tumorigenesis.

VEGF is a factor that promotes endothelial cells proliferation thus stimulates angiogenesis [25]. It is also associated with increased recurrence and metastasis [26]. In our study, the levels of VEGF was significantly higher in TNBC and non-TNBC compared to noncancerous tissues. Furthermore, the upregulation of VEGF was significantly higher in TNBC compared to its levels in non-TNBC. These results implies the particular role of VEGF in TNBC development and progression. A correlation between VEGF and shorter relapse-free and overall survival in breast cancer patients have been reported previously [27]. In TNBC, Wang et al reported that monitoring serum VEGF can be an early predictor of response to treatment and patients' survival [28].

VEGF exerts its function by binding to one of its receptors VEGFR1 and VEGFR2 which are considered key members of receptor tyrosine kinases (RTKs). This binding activates several pathways like mitogen-activated protein kinases, phosphoinositide 3-kinases (PI3Ks), and protein kinase B (Akt) [29]. Thus, inhibiting the VEGF-mediated signaling pathways is a promising strategy for developing new targeted therapeutics.

Mounting evidence suggest that epigenetic regulation with miRNAs plays an important role in VEGF expression including miR-21, -10b, -155, -373 [30], -17, and -92 [31]. In the light of this evidence, we investigated the role of miR-206 in the regulation of VEGF pro-angiogenic function. Our results revealed that cell lines transfected with miR-206 mimics experienced a significant down regulation of VEGF. Similar behavior has been reported previously in renal carcinoma [32], colorectal [33] and lung cancers [34]. These key findings indicate that miR-206 can suppress VEGF-mediated invasion and angiogenesis in TNBC. A previous report by Liang et al who was the first to introduce the relation between mi-206 and angiogenesis-related markers including VEGF in TNBC [35].

KRAS, a member of the RAS GTPase family, is a proto-oncogene involved in the regulation of cell proliferation, apoptosis on top of other vital biological functions [36]. In the current study, KRAS was upregulated in TNBC tissues compared to control as well as non-TNBC tissues. KRAS, and other members of the RAS/MAPK pathway, are aberrantly upregulated in TNBC [37]. Building evidence suggests that the overexpression of KRAS and activation of KRAS/MAPK pathway is more prevalent in TNBC than other subtypes of breast cancer [38,39]. In a study conducted by Kim et al, KRAS expression has been associated with promoted cell invasion and mesenchymal features of basal-like breast cancer [40].

Furthermore, our results revealed that the levels of KRAS expression was negatively correlated with miR-206 expression, insinuating the regulatory role of miR-206 on KRAS expression. Owing to its oncogenic activity, KRAS have been a potential molecular target for developing new

therapeutics, however, its direct inhibition have been very challenging [41]. Upon transfection of cell lines with miR-206 mimic, the upregulated expression of KRAS in both triple negative and non-triple negative cell lines decreased significantly. The expression levels of KRAS continued to drop in a miR-206 dose dependent manner. Similar results have been reported in different types of cancer. In pancreatic adenocarcinoma, miR-206 modulated cell proliferation, invasion and lymphangiogenesis in through targeting of several genes including KRAS [42]. Multiple studies have also implicated that KRAS may also be regulated by a number of miRNAs, including miR-16 and -337 in colorectal cancer [43,44], miR-216b in nasopharyngeal carcinoma [45], and miR-30c and -21 in non-small-cell lung cancer, and -134 in breast cancer cells [46]. However, to the best of our knowledge, this is the first study to correlate the expression of KRAS with the modulatory effect of miR-206 in TNBC.

In conclusion, based our combined human tissues and cell line-based investigations we can suggest that targeting VEGF and KRAS by miR-206 can be a highly efficient therapeutic strategy in TNBC.

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