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NF-ϰB is Over-expressed in Breast Cancer Cell Lines, MCF-7 and MDA-MB-231, Following miR-590 Transfection

Research Article

Azar Sheikholeslami, Ph.D. candidate[1] *, Mohammad Nabiuni, Ph.D.[2],Ehsan Arefian, Ph.D.[3], Fatemeh Jamshidi-Adegani, Ph.D. candidate [4]

[1]Department of Animal Biology, Faculty of Biological Sciences, Kharazmi University, Tehran, Iran,
[2]Department of Cell and Molecular Biology, Faculty of Biological Sciences, Kharazmi University, Tehran, Iran, nabiuni@khu.ac.ir
[3]Department of Microbiology, School of Biology, College of Science, University of Tehran, 1417614411, Tehran, Iran, arefian@ut.ac.ir[4] Molecular department, Farzan Pathobiology laboratory, Hamadan, Iran
[4]Department of Molecular Biology and Genetic Engineering, Stem Cell Technology Research Center, 1997775555, Tehran, Iran, fjamshidiadegani@gmail.com

*__Correspondence__: Azar Sheikholeslami P.O.Box: 31979-37551, Department of Animal Biology, Faculty of Biological Sciences, Kharazmi University, Tehran, Iran E-mail: azareslami@yahoo.com Tel: 00989127506863, Fax: 00982634510005
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Summary

Breast cancer is a heterogenous disease, considered as the most common malignancy in women worldwide. Despite all efforts on identifying cancer, there is no definite therapy yet and much more attempts in discovering cancer biology seems necessary. Immunology of tumors declares the relevance of immune system, chronic inflammation and cancer. Many studies have supported the role of NF-ϰB in linking inflammation and tumorigenesis. NF-ϰB as an important actor of Inflammation, plays diverse roles in cancer. microRNAs have been implicated in a number of diseases including a broad range of cancers. miR-590 has been reported to regulate different pathways and affect different types of cancers. Objectives: Regarding that miR-590 plays various roles in different cells and it is down regulated in many cancer cells, we decided to transfect it in MDA-MB-231 (highly invasive) and MCF-7 (poor invasive) cells and trace the expression of NF-ϰB as an effective gene in cancer cells. The effect of miR-590 transfection in breast cancer cell lines on the expression of NF-ϰB was evaluated in this study. Materials and Methods: The primer for miR-590 was designed and used for PCR. The PCR product was extracted and the miRNA gene was cloned into the vector. miR-590-pLenti-III-eGFP vector was transfected into the breast cancer cells. 72 hours after transfection, the expression of miR-590 and NF-ϰB were measeured by qRT-PCR. Results: miR-590 was over-expressed as it was expected to occur and NF-ϰB level showed significant increase 72 hours after miRNA transfection in both cell lines. Conclusion: In this study, the sudden and redundant increase of NF-ϰB followed by miR-590 transfection in breast cancer cells was seen. It seems that miR-590 regulates NF-ϰB expression directly or indirectly. miR-590 sounds to be an effective regulatory factor in breast cancer which requires more studies to investigate its function on cancer and inflammation.

I. Introduction

Inflammation is the process of innate immunity which is triggered by physical, physiological and/or oxidative stress and can activate the canonical NF-κB signaling pathway, which is conserved in all multicellular animals (1). NF-κB, as an important actor of Inflammation, plays diverse roles in cancer. On one hand, during immune defense NF-κB is activated which targets and eliminates transformed cells. This occurs in acute inflammatory processes, where cytotoxic immune cells attack severely against cancer cells. On the other hand, NF-κB is constitutively activated in many types of cancer, in which begins many pro-tumorigenic functions (2).

There are immune-suppressed individuals, e.g. after organ transplantations, who are in higher risk of cancer and it is obvious that the immune system plays an important role against malignant cells. The anti-tumorigenic function of the immune system is known as tumor-immunosurveillance (3), but it is not tight enough to delete all the aberrant cells so that cancer cells can escape from the immune system (4). Both tumor-immunosurveillance and cell escape seem to be characterized by a chronic inflammatory condition and increased activity of NF-κB. NF-κB activation affects on anti-apoptotic genes and upregulate them which results in cell survival. Additionally, NF-κB induces cytokines which regulate the immune response (such as IL-1, IL-6, IL-8 and TNFα), as well as adhesion molecules, that lead to the recruitment of leukocytes to the inflammatory sites (5, 6). The contribution of NF-κB as an important inflammatory agent in cancer initiation and progression is intricate, however, it seems that NF-κB can promote metastasis by up-regulation of matrix metalloproteinases (MMPs), as well as VEGF and its receptors. MMPs loosen the extracellular matrix and make it possible for cancer cells to migrate and VEGF control vascularization of tumors (7, 8).

Breast cancer is the most common malignancy that develops in women worldwide, its incidence continues to rise and it is responsible for high cancer-associated death rates (9). Breast cancer is a heterogeneous disease, which is made up of different subtypes. There is a way of classification of breast cancer which is based on the presence or absence of receptors for the hormones estrogen (ER), progesterone (PR) and human epidermal growth factor 2 (HER2). An important clinical subtype is triple negative breast cancer (TNBC) which is characterized by an absence of ER, PR and HER2 and which therefore lacks common targets used for anti-hormone therapies (10, 11). MDA-MB-231 is a TNBC cell line which is highly invasive, whereas, MCF-7 is hormone responsive and less invasive.

microRNAs (miRNAs) are a class of small (~22 nucleotides) non-coding RNAs that control gene expression by targeting mRNAs and triggering either translational repression or RNA degradation (12). miRNAs are involved in a wide range of physiological and pathological processes, such as embryogenesis, development, cell differentiation, cell proliferation, cell death, aging, immune responses, tumorigenesis, as well as circadian rhythms (13, 14). In addition to their important roles in healthy individuals, microRNAs have also been implicated in a number of diseases including a broad range of cancers, heart disease and neurological diseases. Consequently, microRNAs are intensely studied as candidates for diagnostic and prognostic biomarkers and predictors of drug response (15, 16). Several studies have identified critical roles for miRNAs in breast cancer. In 2005, it was firstly reported about the deregulation of miRNA expression profiles in breast cancer comparing with normal breast tissue. It was shown that the expression of several miRNAs was dependent upon subtypes and clinicopathological features of breast cancer such as hormone receptor status, clinical stage and proliferation index, suggesting that miRNA expression in breast cancer may have diagnostic and prognostic value (17).

Several miRNAs have been identified with prognostic significance, such as miR-210, miR-126, miR-21 and miR-205 (18, 19). Avery-Kiejda et al., identified several miRNAs with prognostic significance in breast cancer, whereas they introduced some miRNAs including miR-590-5p, miR-1308, miR-17, which have not previously been implicated in breast cancer (16). miR-590-5p has been reported to enhance or inhibit cell growth and invasion depending on the cellular context. Actually, miR-590-5p was reported to be down-regulated in six hepatocellular carcinoma cell lines (20, 21).

I. Objectives

Regarding that miR-590 is reported to play various roles in different cells and it is down regulated in many cancer cell lines, we decided to transfect this miRNA in MDA-MB-231 (highly invasive) and MCF-7 (poor invasive) cells and trace the expression level of NF-κB as an effective gene which plays important roles in cancer cells. In this study, the effect of miR-590 on the expression of NF-κB breast cancer cell lines was investigated.

II. Materials & Methods

- **Primer design and plasmid construction**

For miRNA gene extraction, a template should be used and so, human genomic DNA was extracted from human primary cell (here, we used bone marrow derived mesenchymal stem cell) and was applied as a template for miRNA gene PCR. The primer for selected miRNA was designed in order to produce miRNA clones. PCR product was extracted from gel after electrophoresis. The candidate miRNA gene was cloned into the vector pLenti-III-eGFP (MBA Co., Canada). Briefly, the PCR product and pLenti-III-eGFP vector were digested using EcoR I and BamH I and ligated and the ligation product was transformed into DH5α competent cells. Then, miRNA-pLenti-III-eGFP was verified by PCR and sequencing.

- **Cell culture**

In this experimental study, two human breast cancer cell lines (MDA-MB-231 and MCF-7 cells) were purchased from Iranian Biological Resource Center (IBRC). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, USA) supplementing with 10% fetal bovine serum (FBS; GIBCO, USA) and 1% Penicillin/Streptomycin (GIBCO, USA) and incubated at 37°C, 5% CO2 in a humidified atmosphere (Memmert, Germany). The cells were plated approximately 24 hours before transfection at optimal confluency of about 70%. In transfection day, the cells were divided into 2 groups: test group in which the cells were transfected with miR-590-5p,

and control group which received vector without any miRNA.

· **Transfection**

Serum-free medium as diluent and plasmid DNA, as well as X-tremeGENE HP DNA Transfection Reagent (Roche, Germany; cat# 06366236001) were used to transfect the cells. According to the manufacturer's instructions, cells were transfected with miR-590-pLenti-III-eGFP or control vector. Briefly, diluent was placed in a sterile tube and plasmid was added and gently pipeted to mix. Then, X-tremeGENE HP DNA Transfection Reagent was added to the diluted DNA and incubated for 15 min at +15°C. This transfection complex was added to the cells in a drop-wise manner. The wells were gently shaked and swirled to ensure even distribution over the entire plate. The cells were incubated for 72 hours and then the expression level of miR-590-5p and NF-ϰB was measured by quantitative reverse transcriptase PCR (qRT-PCR) (ABI StepOnePlus Real-Time PCR System, USA).

· **Quantitative reverse transcriptase PCR (qRT-PCR)**

Total RNA was isolated from the cells 72 h after the transfection of miR-590-pLenti-III-eGFP using RNX-Plus (Sinaclon, Iran). It was reversely transcribed into cDNA using 2-step RT-PCR kit (Vivantis, Malaysia) to measure the expression level of NF-ϰB according to the manufacturer's instruction. Whereas, cDNA synthesis for miR-590 was performed with the same kit (Vivantis, Malaysia) with the difference of using RT primer instead of oligo d(T), as well as using SNORD47 as the internal control for miRNA rather than beta-actin for NF-ϰB. Real time RT-PCR was performed using RealQ Plus 2x Master Mix Green (Ampliqon, Denmark). The expression of miRNA and NF-ϰB was normalized using internal controls SNORD47 and beta-actin, respectively, and the 2-ΔΔCT method was used to examine the relative expression levels in treated and control cells. Each test was run in triplicate. The primers' sequences are represented in the Table 1.

III. Statistical Analysis

The one-way ANOVA and InStat software were used to determine the statistical significance of differences between the values for the experimental and control groups. Data were expressed as means ± standard error (SEM), and the results were taken from at least three independent experiments, performed in triplicates. Values of $p \leq 0.05$ were considered statistically significant.

IV. Results

Two breast cancer cell lines, MCF-7 and MDA-MB-231, were transfected with miR-590-pLenti-III-eGFP vector containing GFP as a fluorescent dye which enabled us to trace the miR-590 entrance into the cells. Immuno-fluorescent microscopy images showed remarkable presence of miR-590 in the cells (Fig. 1). The presence of miR-590-pLenti-III-eGFP in the cells after transfection could be estimated to be about 70-80% by immuno-fluorescent images. Also, to confirm the miR-590 entrance into the cells in addition to the Immuno-fluorescent microscopy images, relative real time qRT-PCR was performed which showed significant over-expression of miR-590-5p in both transfected cell lines (Fig. 2). As a result of miR-590 transfection into the cells, the expression level of NF-ϰB was affected and a significant over-expression was seen in both transfected cell lines (Fig. 3). The relative expression level was measured with 2-ΔΔCT method in both miR-590 and NF-ϰB. P value of < 0.05 was considered significant.

V. Discussion

Recently, many studies have supported the role of NF-ϰB in linking inflammation and tumorigenesis (22, 23). Many pro-inflammatory cytokines and chemokines, such as TNF-α, IL-1, IL-6, and IL-8, produced upon NF-ϰB activation, are associated with tumor development and progression (24). NF-ϰB can also be activated by many oncogenes and chemopreventive chemicals to play a crucial role in tumorigenesis and tumor progression (25). Recently, somatic mutation has been implicated to be causative in NF-ϰB activation in cancers. Several NF-ϰB -relevant genes are mutated in multiple myeloma (26), whereas, NF-ϰB activation mechanisms in solid tumors have not been well understood (27). A recent study on breast cancer revealed mutations in NF-ϰB, the upstream kinase IKK2, as well as the inhibitors IϰBα and IϰBε (28). Studies on transgenic mice unveil a direct contribution of the NF-ϰB pathway to the initiation and progression of various solid tumors. For instance, in inflammation associated colon cancer, IKK2 induced NF-ϰB within intestinal epithelial cells has a pivotal role for tumor formation. In general, aberrant NF-ϰB activity seems to have an important role as co-factor in solid tumors by acting as survival factor for transformed cells (29).

Many questions still remains unanswered about the breast cancer origination. Recently, microRNAs are identified as the causative factor whose expression levels vary in different stages of breast cancer and they play an important role in disease progression and metastasis (30, 31). Actually, the levels of miRNAs are regulated by different factors such as hormones that render the miRNAs as a secondary post transcriptional regulation which further alter the pathways in breast cancer (32). Up or down regulation of miRNAs can mediate the pathway of a gene regulation (33). Several studies have supported the role of miRNAs in cancer initiation and progression, as well as in physiological processes such as immune responses, cell proliferation, cell death, and inflammation, which are also known to be mediated by NF-ϰB. This has led many scientists to investigate the convergence of miRNAs and their target genes with NF-ϰB pathways that are critical to tumor development and progression (34).

Several miRNAs such as miR-9, miR-21, miR-143, miR-146 and miR-224 are known as transcriptional targets of NF-ϰB (35). These miRNAs can mediate the activity of NF-ϰB by targeting some of the upstream signaling molecules or members of the NF-ϰB family themselves. In addition, NF-ϰB can induce the synthesis of proteins that regulate miRNAs such as NF-ϰB-dependent induction of Lin28, which prevent the maturation of let-7 miRNAs, a family of miRNAs that is often down-regulated in cancer and which act as tumor suppressor. Let-7 miRNAs target IL-6, thus a reduction of let-7 leads to higher levels of IL-6 and further activation of NF-ϰB generating a positive feedback loop (36). In addition to

regulating miRNAs, NF-ϰB activity itself is regulated by different miRNAs that suppress NF-ϰB family members directly or some of the upstream signaling molecules (37).

miR-590-5p was reported to be down-regulated in six hepatocellular carcinoma cell lines, while S100A10 was up-regulated. S100A10 (from S100 protein family, a highly conserved group of low-molecular weight EF hand calcium-binding proteins) plays a crucial role in the recruitment of macrophages to the tumor site (20). Hepatocellular carcinoma and neoplastic tissues from clinical samples were analysed and it was shown that S100A10 contents were increased in cancer tissues. Over-expression of miR-590-5p, as well as decreased in S100A10 expression caused inhibition of cell growth and induction of cell cycle G1 arrest in HepG2 cells. Furthermore, the over-expression of miR-590-5P decreased the expression of Wnt5a which is considered as an oncogene. Another oncogene, c-myc, was also reduced, contributing to the inhibited proliferative activity of HepG2. Additionally, a decrease was seen in Cyclin D1 expression level as an important regulator of G1 to S-phase transition, which may mediate G1 arrest of HepG2 cells (38). On the other hand, Caspase 3 was increased by over-expression of miR-590-5p, indicating that miR-590-5p may also enhance the apoptosis under the experimental conditions. All these changes may be responsible for the inhibitory effect of miR-590-5p on the growth of HepG2 and 590-5P may be a potential target molecule for the therapy of hepatocellular carcinoma, though further studies are required to confirm the mechanisms (39).

On the other hand, it was shown that the expression of pre-mir-590 was down regulated in human breast cancer and this could be regulated by its target ATF-3 in human breast cancer cells. ATF-3 is a stress response gene product, which is involved in breast cancer metastasis, along with cell invasion and proliferation. It was reported that there is a negative feedback regulation of expression between pre-mir-590 and ATF-3 in human breast cancer cells (40).

VI. Conclusion

Generally, excessive activity of NF-ϰB seems to play a significant role as a survival factor in solid tumors. The effect of miR-590 on NF-ϰB in breast cancer cell lines has not been studied previously. In this study, the sudden and redundant increase of NF-ϰB followed by miR-590 transfection in breast cancer cells was seen, which requires more studies to investigate the miR-590 function on cancer and inflammation. It seems that miR-590 regulates NF-ϰB expression directly or indirectly, which is important to be noted in identifying tumor biology and cancer therapy. Actually, it requires more studies to investigate the miR-590 function on cancer and inflammation.

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Table 1. Details of primers' sequences

Primer Name	Primer Sequence
RT 590-5p	GGTCGTATGCAGAGCAGGGTCCGAGGTATCCATCGCACGCATCGCACTGCATACGACCCTGCA
590-5p- F	GCCGAGCTTATTCATAAAAG
590-5p- R	GAGCAGGGTCCGAGGT
RT SNORD47	GTCGTATGCAGAGCAGGGTCCGAGGTATTCGCACTGCATACGACAACCTC
SNORD47- F	ATCACTGTAAAACCGTTCCA
SNORD47- R	GAGCAGGGTCCGAGGT
H-NFKB1-F	GTGCTGGAGTTCAGGATAACC
H-NFKB1-R	GTGGATGATTGCTAAGTGTAAGAC
H-beta Actin-F	CTTCCTTCCTGGGCATG
H-beta Actin-R	GTCTTTGCGGATGTCCAC

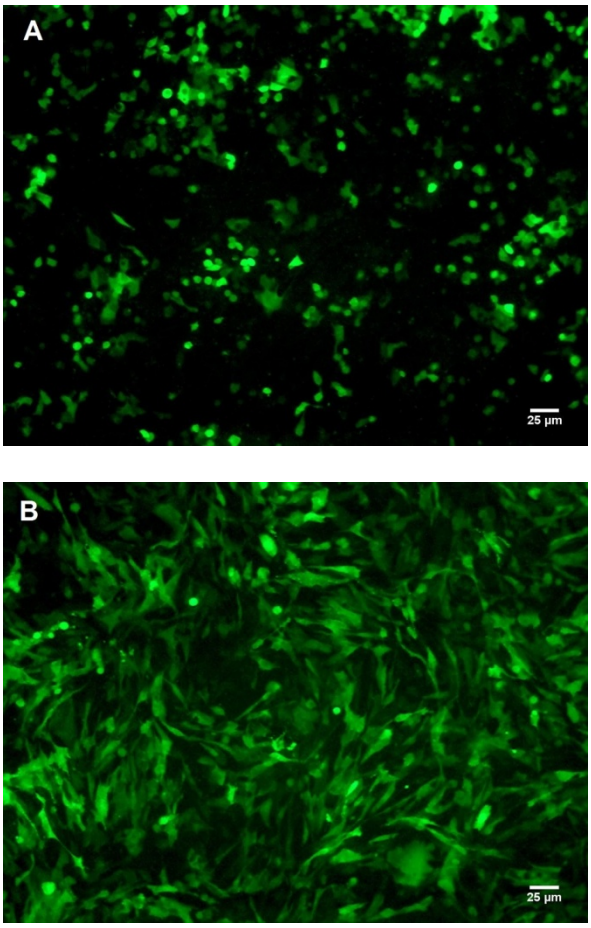

Fig 1. Two breast cancer cell lines transfected with miR-590-pLenti-III-eGFP; A: MCF-7 cell line; B: MDA-MB-231 cell line.

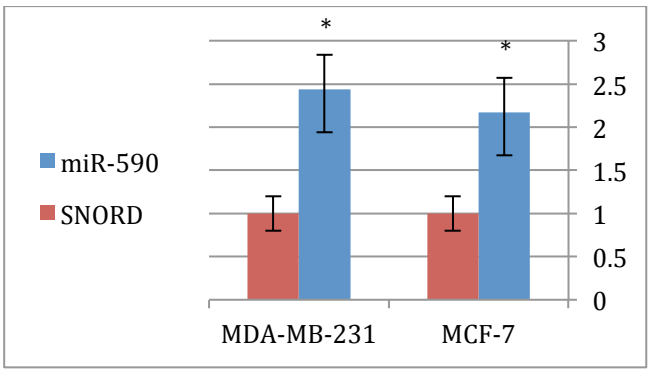

Fig 2. Relative quantification of miR-590 by real-time RT-PCR (fold change based on $2^{-\Delta\Delta Ct}$ method). miR-590 shows over-expression in both cell lines, MDA-MB-231 and MCF-7, after transfection. P value ≤ 0.05.

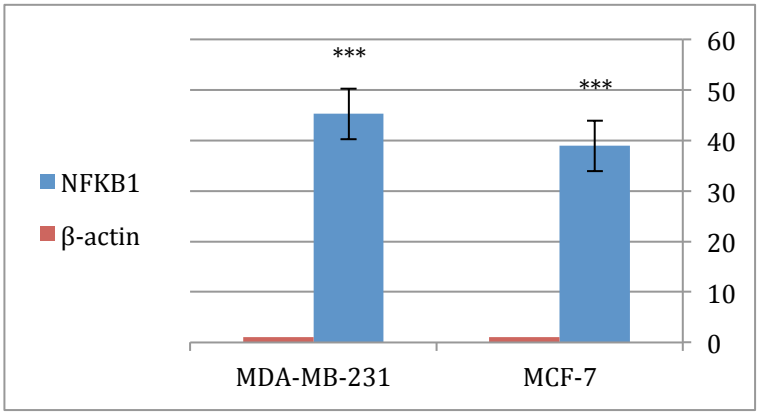

Fig 3. Relative quantification of miR-590 by real-time RT-PCR (fold change based on $2^{-\Delta\Delta Ct}$ method). NFKB1 is over-expressed in cell lines, MDA-MB-231 and MCF-7, after miR-590-5p over-expression. P value ≤ 0.05.

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