The Effect of endurance training on expression of miR-21 and its downstream in breast cancer bearing mice

Sadegh Amani-Shalamzari[1*], Hamid Agh-Alinejad[2], Shaban Alizadeh[3], Shirin Shahbazi[4]

1. Department of Exercise Physiology, Faculty of Sports Science, Kharazmi University, Tehran, Iran
2. Department of Exercise Physiology, Faculty of humanities, Tarbiat Modares University, Tehran, Iran
3. Hematology Department, Allied Medical School, Tehran University of Medical Sciences, Tehran, Iran
4. Medical Genetics Department, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

***Corresponding author:**Tel: +98 9131839372 Fax: +98 2122269547
Address: Department of Exercise Physiology, Faculty of Physical Education of Sports Science, Kharazmi University, Tehran, Iran
E-mail: amani_sadegh@khu.ac.ir
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Abstract

Introduction: Overexpression of oncomir-21 promotes proliferation of breast cancer cells. This study aimed to assess the effect of endurance training on the expression of miR-21 and its downstream, Bcl2 and upstream targets, STAT3 in breast cancer bearing mice.
Materials and methods: After orientation to the environment, breast cancer cells, MC4-L2 were injected to mice and they randomly were categorized into two groups, control (n=10) and training (n=10) groups. Training group performed progressive endurance training 5 days per week for 6 weeks and control group did not perform any exercise. Tumor volume was measured by a digital caliper every week. Finally, the mice were sacrificed; tumor tissue was removed and immediately frozen and kept in -70°C. RNA extraction and cDNA synthesis were carried out by trizol reagent and specific kits and level of genes were measured by quantitative real-time PCR.
Results: Endurance training decreased significantly expression of miR-21, STAT3 and Bcl2 ($P<0.05$). In addition, Tumor volume developed further in control group compared to training group ($P<0.05$). There was significantly positive correlation ($P<0.001$) between miR-21 with STAT3($R=0.66$) and miR-21 with Bcl2 ($R=0.61$)
Conclusion: Endurance training leads to suppress expression of STAT3/miR-21/Bcl2 signaling pathway, thereby involved in slow tumor growth. Therefore, one of the beneficial effects of endurance training on tumor progression in estrogen dependent mouse model of breast cancer is reducing intrtumor anti-apoptotic genes.

Keywords: Estrogen receptor dependent breast cancer, STAT3, Bcl2, miR-21

Introduction

The relationship between exercise training and breast cancer prevention has been proven by numerous epidemiological studies (1), but the involved mechanisms are not fully explored. The beneficial effects of exercise training on breast cancer prevention are complicated and multi-factorial. Recent studies have suggested that a decrease in inflammatory factors associated with exercise training could be one of these mechanisms (2, 3).
Animal models or experimental research is the best method for investigating potential mechanisms of exercise training during cancer. In animal models, type of tumor, type and intensity of exercise, and other

interventions within tumor microenvironment can be controlled precisely. Moreover, the type, intensity, and ideal volume of exercise training to design a training program can be determined with animal models (4). Most studies using animal models of breast cancer have used tumors chemically induced in mice or rats, and have reported positive or negative effects of exercise training (5). Animal models do not reflect human cancer properly. It appears that cancer cell lines have relatively more stable results and simulate human models accurately. The luminal A and B as estrogen receptor-dependent cell lines are the most common subtypes among human breast cancers (6). Therefore, using estrogen receptor-dependent cell lines in animal models is a proper opportunity to investigate human cancers. MC4-L2 is an estrogen receptor-dependent cell line (7), and research on it provides a good opportunity to examine intra-tumor changes following exercise training.

The exploration of MicroRNAs (miRNAs) as small, endogenous, 20-25 nucleotide RNA molecules has created a new insight into the molecular mechanism of cancer pathology(8). A research revealed that over 50% of miRNA genes were in the cancer-related genomic regions(9). Thus, it is suggested that miRNAs are involved in human cancer pathology. Each miRNA knocks out or activates a gene target at transcription, translation, and post-translation level (10). Recent studies have reported that miRNAs are involved in cell growth and apoptosis and may be involved in the formation of cancer cells. Therefore, miRNAs can be the cancer treatment target. The expression of miRNAs varies in different tumor types. Iorio et al. (2005) studied miRNA profiles in healthy and cancerous breast tissues, and found that miRNA expression varied in the both types of the tissues (11). Studies have shown that chronic inflammation is one of the mechanisms, which can modify miRNA expression (12). Chronic inflammation disrupts the expression of oncogenes and tumor suppressors to promote neoplastic transformation (13). Chronic inflammation also leads to the production of some pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α). IL-6 activates the signal transducer and activator of transcription 3 (STAT3)(14), which causes to increase the expression of oncogenes and oncomiRs, particularly miR-21(15). MiR-21 connects several targets together and induces morphological deformation (16). Studies have shown that miR-21 expression as an oncomiR increases in various types of cancer such as pancreatic, colorectal, lung, brain, lymphoma, prostate, colon, gastric, head, neck, and breast cancer (16-18). The upregulation of miR-21 in tumor tissues correlates positively with proliferation and metastasis(18). The increased miR-21 expression is involved in multiple carcinogenic processes such as apoptosis inhibition, increased cell proliferation, and cell growth stimulation (16). Yan et al. (2008) suggested that miR-21 expression increased simultaneously with cancer progression from the stage 1 to the stages 2 and 3 and metastasis. Thus, they concluded that miR-21 could be used as a biomarker of molecular diagnosis for breast cancer and its progression (19). Recent studies have identified programmed cell death protein 4 (PDCD4), tropomyosin-1 (TPM1), and B-cell lymphoma (Bcl-2) as direct targets of miR-21(20).

Bcl-2 as an anti-apoptotic factor contributes to cancer development. Bcl-2 is overexpressed in distinct types of human tumors including prostate, colon, leukemia, and skin cancer, and is considered as a biomarker of molecular diagnosis for cancer (21, 22). Bcl-2 is an anti-apoptotic protein belonging to a group of related proteins, which are key regulators of apoptosis or programmed cell death. The knockout and overexpression of miR-21 decreases and increases Bcl-2 mRNA expression, respectively (16). A study suggested that miR-21 caused to increase

Bcl-2 indirectly by suppressing genes negatively regulating Bcl-2 expression (16). However, some studies have shown that miR-21 is able to directly affect Bcl-2 expression by connecting to its 3'UTR (16, 23). Thus, Bcl-2 upregulation with miR-21 has been observed in breast cancer cells (16).

There is great evidence suggesting that exercise training can reduce the risk of different types of malignant cancers such as colon, breast, prostate, endometrial, and lung cancer (24, 25). In some rodent studies, reduced tumor volume has been reported following exercise training, but its exact mechanism is not fully understood (4, 25, 26). Reducing exercise training-related inflammatory factors has been proposed as the positive effect of regular exercises (4, 26). In addition, it has been reported that exercise training can decrease circulatory miRNAs such as miR-21(27). Two studies reported that interval training along with hormone administration led to decreased miR-21 in mice bearing breast tumor (26, 28).

Altogether, intracellular inflammation increases miR-21 expression through various ways, including IL-6/STAT3 pathways. MiR-21 upregulates Bcl-2 mRNA by targeting it, and, as a result, the apoptosis pathway is suppressed. Therefore, the situation for the progression and metastasis of cancer cells is provided. Several studies have reported the reduction of tumor volume with exercise training (4, 26, 28, 29), but its exact potential mechanism is unknown. We hypothesized that endurance training would modify the apoptosis signaling pathway, which, in turn, would suppress cancer cells. Therefore, this research aimed to investigate the effects of endurance training on anti-apoptosis miR-21 expression and its upstream Bcl-2 and downstream STAT3 targets in mice with breast cancer.

Materials and methods

Cell culture and tumorigenicity

The mice ductal mammary tumor cell line (MC4-L2) that are estrogen receptor positive (ER+) was cultured in T-75 flask and DMEM/F-12 with 15 mm HEPES, Buffer, Glutamin, Penicillin 100 µg/ml, Streptomycin 100 µg/ml and FBS 10% (Gibco BRL, Life Technologies) and 10 nM Medroxy Progestrone Acetate (Sigma, Ontario, Canada). The cells were dissociated from the plate bottom by Trypsin enzyme 0.025%, rinsed with PBS and enzymatically neutralized by using 10% FBS. All the contents of the flask were poured into the falcon tubes and centrifuged in 1200 rpm for 3-5 min, then supernatant was decanted, and cellular plate was dissolved into the containing FBS 10%. The cell viability was determined by Trypan blue and hemocytometer respectively. Cell suspension was prepared with a density of 10 million cells per ml in FBS buffer. The mice initially anesthetized with injection of a dose of 100 mg/kg ketamine and 10 mg/kg xylazine intraperitoneally, and then one million cells were injected subcutaneously in the right flank near to the upper part or rear foot of the animals.

Animals

The present study was experimental research. Twenty female mice Balb/c (6 to 8 weeks old) weighing 13-15 g were purchased from Pasteur institute and, transferred to the animal house in Tarbiat Modares University. The mice were adopted to a 12 hours light-dark cycle for one week. The room temperature was maintained between 22-24°c and the level of humidity was 45%. After a week of orientation in the environment and training on treadmill, cancer cells (MC4-L2) injected to the mice and the protocol started at 10 days later. Mice were randomly divided into two groups, include control group (CG, n=10) that did not perform training, and the exercise group (EG, n=10) that performed moderate intensity endurance training for 6 weeks, 5 days per week (Table 1). The surfaces of treadmill lines were completely covered with black

tape and two black sponges were placed in back and front of each line. Mice were trained without any electric shocks. This study has conducted according to the relevant national and international guidelines of Weatherall report, and Institutional Animal Care and Use Committee of Tarbiat Modarres University.

Training protocol

At the end of the orientation week, mice were tested for maximal effort. Following 5 minutes of warm-up at 10-12 m/min, treadmill speeds increased 1.8 m/min from the initial speed (12 m/min) every 2 minutes. The maximum speed, which the mice were unable to run, was recorded. The maximum speed was 32 ± 1.5 m/min. The exercise begun with 50% of maximal speed and increased 5% every week and reach at 75% of maximum speed at sixth week. Mice were trained 5 days per week. Moderate intensity was used because it has been reported the moderate intensity training reduced tumor volume and higher intensity greater than 80% of maximum effort increased the tumor volume (30). The duration of endurance training started with 30 min in the first week and increased it 5 min every week.

Weight and food consumed

All animals were weighed weekly using digital scales. Feeding was done using normal food for mice. Consumed food was the difference between the remaining foods from the total food amount, which put into the cage; it was measured weekly.

Tumor volume

the length and width of tumor were measured each week by digital caliper and, the volume of it was measured by using the formula ($V = \pi/6\ (w \times l^2)$(29). The tumor volume was measured in two dimensions, the largest dimension of the tumor was considered as the length (L) and the other dimension (at a 90-degree angle) as width (W) of the tumor. Then, the calculated values of last day was divided by the first day did to estimate the tumor volume rate.

Heart weight and tumor weight

After sacrificing the mice, hearts were removed immediately and heart weight was measured using digital scales following withdrawn the remaining blood. The heart weight to body weight ratio is considered as an indicator of training efficiency (30) and it calculated by dividing the heart weight by body weight. In addition, tumor tissue was removed immediately and weighed it by digital scales.

Total RNA extraction and cDNA synthesis

Following sacrificing of the mice, the tumor was removed immediately, and after removal of central part (necrosis portion), the supernatant portion of the tumor was freezed in liquid nitrogen at -70°c in the laboratory. Total RNA was extracted from 50-100 mg of tumor using Trizol solution (Invitrogen, USA), and cDNA synthesis (Qiagen, cat:205311 Germany) according to the manufacturer's instructions. cDNA synthesis of microRNA was carried out according to the kit protocol (stratagene, cat:600-583, USA). The STRATAGENE Kit contains miRNA1st-strand cDNA and miRNA QPCR MASTER MIX. The miRNA 1st-strand cDNA synthesis kit was used to elongate miRNAs in a polyadenylated RNA into QPCR-ready cDNA. The gene and microRNA sequences were extracted from NCBI gene bank and microRNA gene bank (www.mirbase.org) respectively. The used forward primer for miR-21 was UAGCUUAUCAGACUGAUGUUGA; the universal reverse primer was served as the downstream primer in the QPCR reaction; forward primer U6 as housekeeping gene was GCGCGTCGTGAAGCGTTC and reverse sequence was GTGCAGGGTCCGAGGT; forward and reverse sequences for STAT3 was ACCCAACAGCCGCCGTAG and CAGACTGGTTGTTTCCATTCAGAT;

forward and reverse sequences for Bcl-2 was CTGCACCTGACGCCCTTCACC and CACATGACCCCACCGAACTCAAAGA; and forward and reverse sequences for GAPDH was TCAACAGCAACTCCCACTCTTCC and ACCCTGTTGCTGTAGCCGTATTC.

Real- time PCR

To determine the mRNA relative expression of BCL2 and STAT3, qRT-PCR was carried out by using SYBR green dye. The thermal cycling program for STAT3 and Bcl-2 was as follow: 94°C for 3 min followed by 30 cycling of 94° C for 30 second, 54°C for 60 second, and 72°C for 30 second. GAPDH mRNA was applied for normalization of the gene expression analysis. The thermal cycling program for miR-21 was: 94°C for 10 min followed by 45 cycling of 94°C for 10 second, 60°C for 15 second and 72°C for 20 second.

Statistical analysis

Data presented as mean (SD). Statistical analysis and measuring gene expression were performed by REST software with Mann-Whitney nonparametric tests. Independent T-test were used to analyze the data of the tumor volume rate, the repeated measure ANOVA was used to analysis the body weight and consumed food. Pearson correlation coefficient and curve fitting plots were used to determine the correlation between variables. All analyses were performed with SSPS statistical software (version 19) with the significance level set at $P<0.05$.

Results

Weight of mice and food consumed

Table 1 presents the mice body weight and the amount of food consumed during the research. The repeated measure ANOVA showed there was no significant difference in body weight between two groups ($F=2.2$, $P=0.21$). We observed a decreasing and increasing trend for food intake in CG and EG respectively. Results of repeated measure ANOVA showed food intake during implementing the protocol differed significantly between two groups ($F=6.4$, $P=0.04$).

Table 1. The body weight and food consumed during the research protocol.

	Group	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Body weight (g)	Control	17.7 ±0.9	18.1±0.8	18.4± 1.2	19.2±1.1	19.5±1.1	20.5±1.2
	Exercise	17.7±0.7	17.9±0.8	18.0±0.8	18.4±0.8	19.5±1.2	19.8±1.2
Food consumed (g)	Control	165.4	168	164	160.5	153	142.6
	Exercise	164.5	170.3	168.8	170.4	167.7	170.4

The tumor volume and tumor weight

Finding indicates the rate of tumor development in EG was slower than the control group. Figure 1 shows rate of tumor development during the 6 weeks of implementation of the research protocol. The final amount and rate of tumor development in CG was higher than EG. By dividing the tumor volume in sixth week by the first week, the tumor development ratio was obtained. Independent t-test showed there was a significant difference between two groups in tumor volume ($t=6.6$, $p=0.001$) and tumor weight ($t=7.6$, $p=0.001$). The mean of the ratio for EG was 4.8 ± 0.5 and for CG was 7.4 ± 1.7. Therefore, the tumor development rate in EG was lower than CG. In addition, the tumor weight in CG was greater than EG (Table 2).

Table 2. The mean (SD) of heart weight and heart to body weight ratio.

	Heart weight (g)	Tumor weight (g)	Heart-to-body weight ratio
Control group	0.091 ± 0.021	2.83 ± 0.56	0.00445 ± 0.00066
Exercise group	0.118 ± 0.018	1.21 ± 0.57	0.00617 ± 0.00095

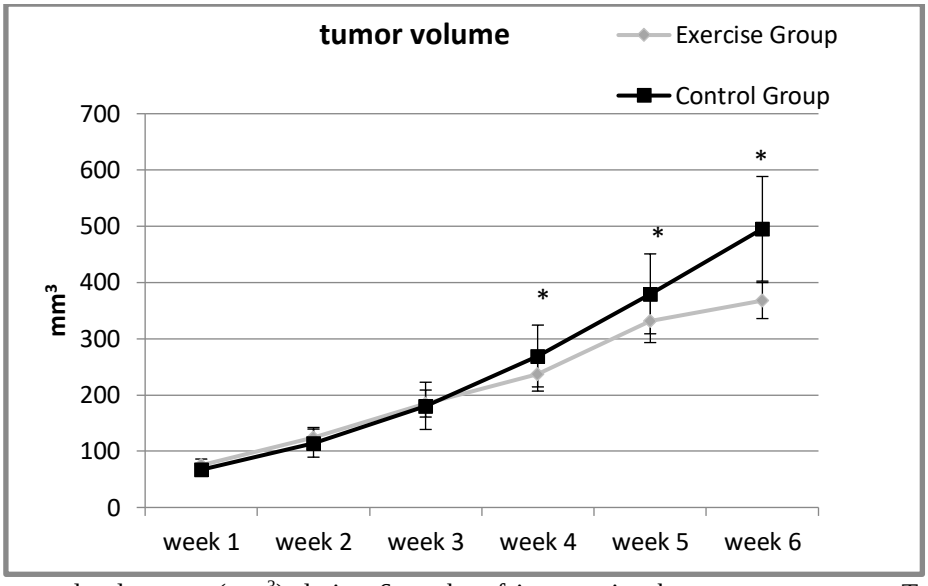

Figure 1. Tumor development (mm^3) during 6 weeks of intervention between two groups. Tumor volume developed further in control group compared to training group. The differences appeared after fourth week.

Heart weight and heart to body weight ratio

Following training protocol, there was a significant difference between EG and CG in heart weight (t=6.5, p=0.001) and heart-to-body weight ratio (t= 4.5, p=0.001). The average of heart weight in EG was higher than CG, which subsequently increased the heart-to-body weight ratio.

Expression level of miR-21

The results of a quantitative Real-Time PCR technique were analyzed using the REST software. There was a significant reduction in the gene expression of miR-21 in EG compared to CG (p=0.001, the standard error 0.605-0.084), the expression level of miR-21 in EG compared to the CG was 0.21.

The expression level of BcL2 and STAT3

We found a significant decrease in expression of Bcl-2 (P=0.014, the standard error 0.975-0.110) and STAT3 (P=0.001, the standard error 0.012-0.246) in EG compared to CG. The expression level of Bcl-2 and STAT3 in EG compared to CG was 0.325 and 0.051 respectively, indicating a reduction in the expression of these genes.

Non-linear correlation of STAT3 and BCl2

Pearson correlation coefficient and curve fitting plots were used to determine the relationship between variables. Data showed that there was a relatively strong non-linear correlation between miR-21 with STAT3 and Bcl-2 genes. Figure 2 & 3 presented the fitting line plots of miR-21 with Bcl-2 and STAT3. R square for the relationship between miR-21 with Bcl-2 and STAT3 was 0.606 (p=0.001) and 0.657 (p=0.001) respectively. The finding suggests that expression of Bcl-2 and STAT3 correlated directly with miR-21.

Discussion

The main finding of this study was the reduced tumor volume in the exercise group. The mechanisms of positive effects of exercise training on reducing tumor volume are multifactorial and unclear. The finding of this study showed that endurance training could modify the apoptosis pathway in breast tumor cells. In addition, the finding showed that there was a direct correlation between miR-21 expression and mRNA expression related to Bcl-2 and STAT3. Thus, endurance training with the help of the anti-inflammatory effect can reduce inflammatory factors within tumor

cells such as STAT3. Thus, endurance training can suppress the STAT3/miR21/Bcl-2 pathway, and also, it can modify the apoptosis pathway by decreasing anti-apoptosis miR-21 expression and its target gene, Bcl-2. Therefore, reducing the gene expression of the cancer progress signaling pathway with endurance training is suggested as one of the effective mechanisms in the management of estrogen receptor dependent of breast cancer.

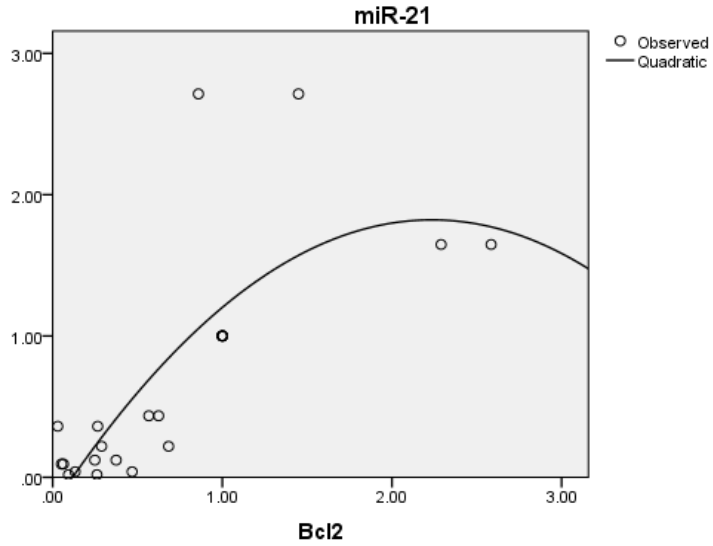

Figure 2. Curve fitting of miR-21 and Bcl2. There was a quadratic equation between expression of miR-21 and Bcl-2, which is presented in the figure.

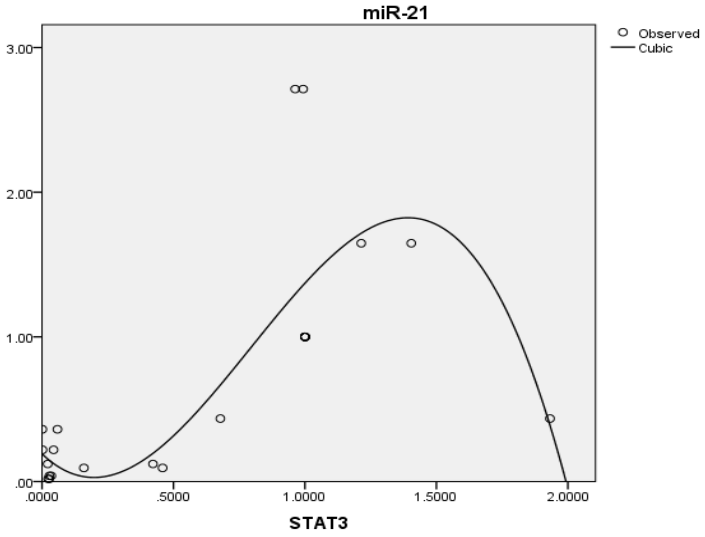

Figure 3. Curve fitting of miR-21 and STAT3. There was a cubic equation between expression of miR-21 and Bcl-2, which is presented in the figure.

Many studies have reported slow tumor volume progress as a result of exercise training, although its mechanism is not fully understood (4). In our study, we also observed that a six-week endurance training led to slow tumor volume progress. The data of the study showed that tumor volume was smaller in EG than in CG in the sixth week. There was a significant difference in the tumor growth ratio between the two groups. Murphy et al. (2011) reported that a 20-week endurance training led to slow tumor volume progress compared to the control group. In addition, they observed

decreased tumor volume from the 17th week to the 20th week (4). They considered that the decrease in endurance training-related inflammatory factors such as IL6 and MCP-1 led to tumor reduction. Moreover, Zielinski et al. (2004) claimed that tumor volume reduction after six weeks of endurance training was attributed to the decreased density of intracellular immune cells (31). In another study, Abdalla et al. (2013) reported a shift in cytokine responses to cell-mediated immunity with exercise training in the experimental group compared to the control group (32). According to their research, the cellular mechanisms of exercise training for reducing tumor volume were complex and multifactorial. Based on our finding, we can state that endurance training via reducing inflammatory factors (IL-6 and STAT3) and modifying the apoptosis pathway (miR-21 and Bcl-2) resulted in slowing tumor growth.

Inflammatory factors are involved in tumor development, and the reduction of these factors is an effective mechanism of exercise training (4). Women with high estradiol concentration are at a higher risk of cancer development (33). Studies have shown that estrogen promotes the development of breast tumor cells by stimulating the production and expression of inflammatory factors (34). Estrogen can stimulate intra-tumor NF-kB expression, which can trigger the production of many inflammatory cytokines such as IL-6 and inflammatory genes such as the signal transducer and activator of transcription 3 (STAT3)(15). STAT3 can shift the inflammatory situation to tumor promotion status and evading apoptosis in cancer cells. STAT3 can also connect to several regions of the mir-21 promoter in deformed cells. STAT3 activation in estrogen-dependent epithelial breast cancer cells leads to mir-21 upregulation. In the present study, there was a strong relationship between STAT3 expression and miR-21 expression, showing that increasing STAT3 expression caused increased miR-21 expression. MiR-21 can increase the expression of anti-apoptotic genes such as Bcl-2 and inhibit programmed cell death protein 4 (PDCD4) by attaching to it, which results in apoptotic pathway suppression, and subsequently, cancer development (16). Since more than 50% of miRNA genes are in the cancer-associated genomic regions (35), it is suggested that miRNAs are involved in cancer pathology in humans. Recent research has targeted miRNAs, especially miR-21 as a cancer indicator, to manage breast cancer (36). This study showed that endurance training led to decreased miR-21 expression; therefore, endurance training can suppress miR-21 signaling.

A study reported Bcl-2 upregulation with miR-21 in human estrogen-dependent breast cancer cells (MCF-7). Bcl-2 is anti-apoptotic protein and belongs to a group of complex proteins that are key regulators of apoptosis or programmed cell death (21). The overexpression of Bcl-2 has been recognized in many malignant cancers, including prostate, colon, skin, leukemia, and breast cancer, and thus, it is considered as a diagnostic cancer biomarker (21, 37). Si et al. (2006) reported lower levels of the Bcl-2 protein in MCF-7 cells injected with anti-miR-21(16). They showed that other apoptotic proteins such as p53 did not change. Therefore, they concluded that apoptosis induction with anti-miR-21 was partly related to Bcl-2 upregulation. In addition, Bcl-2 expression decreased in cells injected with anti-miR-21. Thus, it appears that miR-21 is involved in inhibiting apoptosis in tumor cells via regulating Bcl-2 expression. A strong correlation was found between miR-21 expression and Bcl-2 expression in this study, meaning that increasing miR-21 expression caused to increase Bcl-2 expression. Since endurance training decreases miR-21 expression, reduction in Bcl-2 expression was evident.

Conclusion

Overall, inflammation with STAT3 upregulation is involved in breast tumor

development. STAT3 can increase miR21 expression as an oncomiR. Endurance training has anti-inflammatory effects and decreases inflammatory factors in tumors. The findings of the current study showed that endurance training could decrease miR-21, STAT3 and its target, and Bcl-2. Therefore, one of the effective mechanisms of exercise training in breast cancer treatment can be apoptosis modulation within tumor tissues. Therefore, patients with estrogen-dependent breast cancer are recommended to perform endurance training with specific considerations. Endurance training suppresses the expression of the STAT3/miR-21/Bcl-2 signaling pathway, and thus, is involved in slowing tumor growth. Therefore, one of the beneficial effects of endurance training on tumor tissues in the estrogen-dependent mouse model of breast cancer is reducing intra-tumor anti-apoptotic genes.

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