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Effects of exercise training together with tamoxifen in reducing mammary tumor burden in mice: Possible underlying pathway of miR-21


a Ischemic Disorders Research Center, Golestan University of Medical Sciences, Gorgan, Iran
b Cancer Research Center, Tehran University of Medical Sciences, Tehran, Iran
c Physical Education Department, Shahed University, Tehran, Iran
d Immunological response research center, Shahed University, Tehran, Iran
e Hematology Department, Allied Medical School, Tehran University of Medical Sciences, Tehran, Iran
f Cancer Models Research Center, Tehran University of Medical Sciences, Tehran, Iran
g Medical Genetics Department, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
h Physiology Department, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran
i Pathology Department, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran
j Department of Molecular Medicine, Pasteur Institute of Iran, Tehran, Iran
k Medical Genetics Department, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran
l Colon cancer center, South Carolina University, United Stat of America

Corresponding author: Ali Mohammad Alizadeh, Cancer Research Center, Tehran University of Medical Sciences, Tehran, Iran
Zip Code: 1419733141
Tel/Fax: 00982161192501
Mobile: +98912 5790941
aalizadeh@sina.tums.ac.ir ; alizadehtums92@gmail.com
Abstract

Exercise training has an anti-tumor effect and can reduce tumor growth; however, the exact underlying mechanisms of its protective effects are still obscure. MicroRNA (miR)-21 is a predictor in cancer survival, and has a potential use as an indicator of therapeutic outcome in breast malignancies. Forty-eight female BALB/c mice were equally divided into six groups to investigate the effects of interval exercise training with tamoxifen on miR-21 expression and its possible assumed mechanisms in an estrogen receptor-positive breast cancer model. ELISA, immunohistochemistry, western blot, qRT-PCR assays were performed at the end of the study. Tumor size was significantly declined in exercise training and tamoxifen groups compared to tumor group (P <0.05). Expression of miR-21 was significantly down-regulated in trained and tamoxifen treated mice in comparison with tumor group (P <0.05). Exercise training was as effective as tamoxifen treatment in decreasing serum estradiol and ER-α expression (P <0.05). Exercise training and tamoxifen reduced tumor IL-6 levels, NF-kB and STAT3 expressions, and up-regulated TPM1 and PDCD4 expressions (P <0.05). Both exercise and tamoxifen had synergistic effects in reducing miR-21 and Bcl-2, and up-regulating PDCD4 expression. Results showed that interval exercise training may reduce mammary tumor burden in mice through possible underlying pathway of miR-21.
Keywords: Interval exercise training, MiR-21, Tamoxifen, Breast cancer

1. Introduction

MicroRNAs (miRNAs) are non-coding and single-stranded RNAs of ~22 nucleotides, and constitute a novel class of gene regulators that are found in both plants and animals (Esquela-Kerscher and Slack, 2006). They can play the important regulatory roles by targeting mRNAs for cleavage or translational repression (Bartel, 2004) and therefore, can involve in cell differentiation, proliferation and apoptosis in some diseases such as cancer (Chen et al., 2004). MiR-21 is over-expressed in multiple cancer forms and tightly associated with cancerogenesis. Reportedly, miR-21 was over-expressed in the solid tumors such as estrogen receptor (ER)-positive breast cancers (Iorio et al., 2005). It can be a predictor in cancer prognosis, and has a potential to be used as an indicator of therapeutic outcome in many cancerous states such as breast malignancies (Yan et al., 2008). MiR-21 can act through inhibition of apoptosis (Chan et al., 2005), promotion of cell proliferation (Roldo et al., 2006) and subsequent stimulation of tumor growth (Si et al., 2006). Bye et al. (2013) showed an association between low maximal oxygen consumption and high levels of circulating miR-21. They also showed that miR-21 was increased in women with low VO₂max compared to women with high VO₂max (Bye et al., 2013). Nielsen et al. (2014) found that endurance exercise training decreased the level
of miR-21 in blood circulation where acute exercise had no effect on miR-21 expression (Nielsen et al., 2014). Moreover, increased expression of estrogen receptor-α (ER-α) was associated with an increased expression of miR-21 (Iorio et al., 2005).

Exercise training also activates essential intracellular mediators during exercise regimen such as apoptosis, angiogenesis (Zhang et al., 2010) and inflammation (Davidson-Moncada et al., 2010). IL-6 promotes tumor growth by up-regulating anti-apoptotic and angiogenic proteins in tumor cells (Heinrich et al., 1998; Trikha et al., 2003). Therefore, IL-6 may stimulate proliferation of breast cancer cells through estrogen production (Honma et al., 2002). Exercise first increases IL-6 plasma level, and then rapidly reverses it towards the pre-exercise levels. This may play a role in exercise adaptation (Pedersen & Fischer, 2007). However, regular and endurance exercise has an anti-inflammatory effect, and can decrease IL-6 level (Petersen & Pedersen, 2005), hence reducing inflammation and subsequently cancer development (Murphy et al., 2011; Shalamzari et al., 2014). Cytokines can also modulate miR-21 expression. For example, IL-6 induces the expression of miR-21 in a STAT3-dependent manner (Loffler et al., 2007). Therefore, the present study aimed to investigate the mechanisms of interval exercise training and tamoxifen on reducing tumor growth in a typical animal model of breast cancer through possible miR-21 underlying pathways.

2. Materials and methods

2.1. Materials

Mouse mammary adenocarcinoma cell line (MC4-L2) was obtained as a gift from the Buenos Aires University, Argentina (Lanari et al., 2001). Ketamine and Xylazine (Sigma Aldric, USA), polyclonal mouse antiRat/Rabbit Bax and Bcl-2 antibodies (DAKO
Corporation, USA), mouse IL-6 ELISA kit (Abcam, Germany), Estradiol ELISA kit (Cayman Chemical, USA) and Trizol (life technology, USA) were purchased.

2.2. Cell culture

The mice mammary tumor cell line (MC4-L2) was grown in T75 flasks in DMEM/F-12 with 15 mM HEPES buffer, L-glutamine, penicillin 100 μg/ml, streptomycin 100 μg/ml, 10% FBS (Gibco BRL, Life Technologies) and 10 nM Medroxy Progesterone Acetate (Sigma Chemicals, Ontario, Canada). The cells were detached by 0.025% trypsin, rinsed with PBS and enzymatically neutralized by 10% FBS and finally centrifuged in 1200 rpm for 3-5 min. The cell viability was determined by Trypan blue and hemocytometer, respectively (Lanari et al., 2001).

2.3. Animals

Animal studies have conducted according to the relevant national and international guidelines of Weatherall report, and Institutional Animal Care and Use Committee of Tehran University of Medical Sciences. Inbred female BALB/c mice (6-8 weeks old) were purchased from Pasteur Institute of Iran, and maintained under 12-h dark and light cycle, with free access to food and water.

2.4. Tumorigenicity

MC4-L2 cells were trypsinized and re-suspended in 10-fold excess culture medium. After centrifugation, cells were re-suspended in a serum-free medium. Prepared cells (1×10^6 cells in a final volume of 0.1 ml) were inoculated in the right flank near to the upper part of rear foot of the animals under anesthesia with Ketamine (100 mg/kg) and Xylazine (10 mg/kg).

2.5. The study design
Forty-eight mice were equally divided into six groups (I) control (C); healthy animals with neither tumor induction nor exercise training protocol, (II) exercise training (EX); animals under exercise training protocol for 5 weeks without tumor cell injection, (III) tumor induction (T); animals with breast tumor, (IV) tumor + exercise (T+EX); animals with breast tumor underwent 5 weeks of exercise training protocol after tumor establishment, (V) tumor + tamoxifen (T+TMX); TMX (5 mg/kg, daily) was given via oral gavage for 2 weeks after tumor establishment, and (VI) tumor + tamoxifen + exercise (T+TMX+EX) (Fig. 1). At the end of the study, all animals were euthanized 48 h after the last session of exercise training.

2.6. Interval exercise training protocol

Following tumor establishment, animals were divided into six groups, and the body weight and tumor volume were measured. Prior to the initiation of exercise training, mice in exercise groups were assigned to the treadmill for 5 days. Acclimation entailed running at the end of their dark cycle (0700) at gradually increasing speeds (10, 12, 16, and 18 meter/min) and 0% inclination. Following the acclimation, the exercise protocol began at 16-18 m/min, 0% grade, 10-14 min, 5 days/week for 5 weeks (Riggs et al., 2010). No electrical stimulation used, and mice were encouraged to run by a gentle tap on the tail or hindquarters.

2.7. Body weight and tumor size

Animals were weekly weighed and regularly monitored for abnormal sequel. Tumor volume was measured by a digital vernier caliper (Mitutoyo, Japan) on a weekly basis, and reported as cm$^3$ using the following formula (Mohsenikia et al., 2013):

$$V = \frac{1}{6} (\pi LWD),$$

where $L =$ length, $W =$ width and $D =$ depth.
2.8. Blood and tumor tissue collection

To avoid acute exercise response, mice were euthanized 48 h after the last exercise session. During anesthesia period, blood (1.5 ml) was intracardially withdrawn. Animals were finally euthanized using cervical dislocation. Blood samples were then centrifuged for 10 min at 4,000 g, and the serums were collected and stored at –80 °C for further analysis. Tumor tissues were fixed in 10% formaldehyde, passaged and embedded in paraffin. Paraffin blocks were then sectioned (3 μm) and stained with hematoxylin and eosin (Alizadeh et al., 2012b). The slides were studied using OLYMPUS-BX51 microscope and graded according to the Scarff-Bloom-Richardson Scale (Mohsenikia et al., 2013).

2.9. E2 and IL-6 level assays

Serum 17β-estradiol (E2) level was measured by ELISA method according to the manufacturer's instructions (Cayman Chemical, USA). Fresh-frozen tumor tissues (100 mg) were homogenized in 10 volume of an ice-cold buffer containing 50 mM Tris_HCl (pH 7.8), 2 mM potassium phosphate, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM diithiothreitol, 3 mM benzamidine, 1 mM sodium orthovanadate, 10 mM leupeptin, 5 mg/ml aprotinin, and 1 mM 4-[(2-aminoethyl)benzenesulfonyl fluoride] using a motor-driven glass pestle. The homogenate was immediately centrifuged at 12,000 g for 20 min at 4 °C, and the supernatant was removed as the detergent-soluble fraction. Protein concentrations were determined using the Bio-Rad Protein assay with BSA for the standard curve. Samples were stored immediately in aliquots at –80 °C for subsequent ELISA analysis. IL-6 level was measured in duplicate using validate mouse
ELISA kits (Abcam, Germany) with < 2 pg/ml sensitivity. IL-6 concentration was expressed as picograms per milligram (pg/mg).

2.10. Evaluation of ER-α, NF-κB, STAT3, BCL-2, TPM-1 and PDCD4 mRNA expression

Total RNA was extracted from 50-100 mg of tumor tissue using Trizol solution (Invitrogen, USA), and cDNA synthesis (Qiagen, Germany) was performed using Qiagen cDNA synthesis kit according to the manufacturer’s instructions. Briefly, 1 μg of RNA was added to the reaction mixture (gDNA Wipeout Buffer, Quantiscript Reverse-Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-free water) followed by incubation at 42 °C for 15 min. Reverse transcription was terminated at 95 °C for 3 min, and then samples were stored at −20 °C until used for Real-time PCR analysis.

CDNA synthesis of microRNAs (1 μg total RNA as input) was carried out according to the kit protocol (Stratagene, USA). To determine the relative expressions of TPM-1, PDCD4, BCL2, STAT3, NF-kB and ER-α mRNA, qRT-PCR was performed using SYBR Green dye. Thermal cycling program was as follows: 94 °C for 3 min followed by 30 cycles of 94 °C for 0.5 min, 54 °C for 1 min, and 72 °C for 0.5 min. GAPDH mRNA was used for normalization of the gene expression analysis. PCR primers sequence used for the amplification of the protein coding genes has been shown in table 1.

2.11. Real time qRT-PCR for mir-21 detection

Due to their short length, miRNAs are difficult to detect with standard qRT-PCR protocols. Hence, we used specific miRNA QPCR core reagent kit to quantitatively amplify cDNA templates derived from miRNAs within a total RNA population. Initially, miRNA 1st-strand cDNA synthesis kit (Stratagene, USA) was used to elongate miRNAs and produce QPCR-ready cDNA. The targeted miRNA was then amplified and detected
using specific miRNA QPCR core reagent kit (Stratagene, USA). The universal reverse primer was served as the downstream primer in the QPCR reaction and the specificity of the QPCR reaction was provided by the miRNA-specific forward primer.

2.12. *Immunohistochemical examinations*

Paraffin embedded tissues were stained for immunohistochemical assay using avidin-biotin immunoperoxidase method (Mohsenikia et al., 2013). For tumor cell’s apoptotic pathway study, sections were stained with polyclonal mouse antiRat/Rabbit Bax and Bcl-2 antibodies (DAKO Corporation, USA) according to the manufacturer’s instructions. Briefly, paraffin sections were deparaffinized with xylene and rehydrated through a series of descending graded ethanol solutions. Tissues kept in TBS-EDTA buffer and put into a microwave oven for 15 min at 90 °C. Endogenous peroxidase activity blocked by 0.3% H$_2$O$_2$ buffer for 15 min. Biotinylated secondary antibody and avidin-biotin complex with horseradish peroxidase were applied by chromogen 3, 3’-diaminobenzidine (Sigma Chemical) addition. Bax and Bcl-2 positive cells were identified, and color intensity was semi-quantified using the following protocols (Alizadeh et al., 2012a; Mohsenikia et al., 2013):

No staining: 0
Faint/barely staining up to 1/3 of cells: 1
Moderate staining in 1/3 to 1/2 of cells: 2
Strong staining in more than 1/2 of cells: 3

2.13. *Evaluation of TPM-1 and PDCD4 proteins by western blot*

Frozen tissues were homogenized in SDS lysis buffer (25 mM Tris–HCl pH 6.8, 0.8% SDS, 4% glycerol, 2% 2-mercaptoethanol, 0.008% bromophenol blue). Sodium dodecyl
sulfate-polyacrylamide gel electrophoresis (SDS-PAGE 10%) was performed according to the standard procedures. Proteins were then transferred to polyvinylidene difluoride (PVDF) membrane (Roche, USA). Blocking of residual protein-binding sites on the membrane was performed with 5% nonfat dry milk in TBST (50 mmol/L Tris-HCl (pH7.5), 150 mmol/l NaCl, 0.1% Tween-20) during one h. The membrane was then rinsed 3 times with TBST and incubated overnight at 4 °C in TBST either with tropomyosin α antibody (Santa Cruz, USA), a goat polyclonal IgG, at 1:1000 dilution or PDCD-4 antibody (Santa Cruz, USA), a goat polyclonal IgG, at 1:1000 dilution. After overnight incubation, the membrane washed several times for 5 min with TBST and incubated with the secondary antibody peroxidase conjugated goat anti-mouse IgG (Santa Cruz, USA) at 1:1000 dilutions for one h at room temperature. Following another three washes for 5 min with TBST, the membrane was incubated with chemiluminescence detection kit (Pars Tous biotechnology, Iran) for one min and exposed to Kodak radiography transparent film for band intensity checking.

2.14. Statistical analysis

Two-way ANOVA was used to assess main effects of exercise training and tamoxifen, and interaction between training and tamoxifen for all variables in mice bearing model of breast cancer. If significant effects were found, the Bonferroni post hoc test was used to determine the source of the difference. All statistical analyses were performed with SPSS statistical software (version 13) with the significance level set at P<0.05. Data presented as mean ± S.D.
3. Results

3.1. Clinical observation, body weight and tumor size

There were no behavioral changes between animal groups during the course of administration or follow up period. Body weight was measured weekly during the treatment period. Mice in the exercise group had significantly reduced body weight than control group at sacrifice time (Fig. 2A) ($P < 0.05$). Exercise training resulted in lower body weights in T+TMX+EX and T+EXE vs. T group ($P < 0.05$). The average tumor volume was significantly less in T+EX and T+TMX+EX groups than T group at 28 and 35 days after tumor cells injection ($P < 0.05$). Mean final tumor volume reached to approximately 0.25, 0.37 and 1.43 cm$^3$ in T+TMX+EX, T+EX and T groups, respectively (Fig. 2B). There were no signs of metastasis in main organs of both treatment and control groups. No histopathological abnormality was found in major organs such as brain, heart, liver, kidneys and lungs. In tumor tissues, cells with sarcomatoid-type areas showed no morphological signs of epithelial cell invasion into surrounding tissues. Cells were frequently multinucleated. Histological feature depicted a definite carcinomatous growth pattern with solid groups and cords of polygonal cells.

3.2. Serum levels of E2 and ER-α expression

Serum E2 level was significantly lowered in T+EX group than others ($P < 0.05$) (Fig. 3A). Results showed that serum E2 level in EX group was significantly lower than T group ($P < 0.05$). In addition, it was significantly decreased in T+TMX+EX and T+TMX groups in comparison with T group ($P < 0.05$) (Fig. 3A). ER-α mRNA expression was lower in T+EX, T+TMX and T+TMX+EX groups than T group ($P < 0.05$). The lowest ER-α mRNA expression was seen in T+EX (Fig. 3B).
3.3. Tumor IL-6 level and mRNA expression of NF-κB and STAT3

Tumor IL-6 protein level was significantly lower in T+EX group (58.06 ± 16.05 pg/mg) than other groups \((P < 0.001)\) (Fig. 4A). It was also reduced in T+TMX and T+TMX+EX groups than T group \((P < 0.05)\) (Fig. 4A). NF-κB mRNA expression was down-regulated in T+EX, T+TMX and T+TMX+EX groups in comparison with T group \((P < 0.05)\). The lowest NF-κB value was seen in T+TMX+EX group (Fig. 4B). In addition, STAT3 mRNA expression was suppressed in tumors in mice trained and/or treated with tamoxifen \((P < 0.05)\). The lowest STAT3 value was seen in T+TMX+EX group (Fig. 4C).

3.4. Tumor miR-21 expression

Mir-21 expression, as the suppressor of tumor growth inhibitors, was reduced more in T+EX, T+TMX and T+TMX+EX groups than T group (Fig. 5). Moreover, miR-21 down-regulation in T+TMX+EX group (0.056 fold) was found more than T+TMX (0.29 fold) and T+EX (0.64 fold) groups \((P < 0.05)\) (Fig. 5).

3.5. Expression of tumor Bax and Bcl-2 proteins

Immunohistochemical examinations of T+EX group showed high pro-apoptotic Bax protein expression in breast tumor in comparison with T group \((P < 0.05)\). Anti-apoptotic Bcl-2 protein expression was lower after exercise training in T+EX group \((P < 0.05)\) (Fig. 6A). In addition, exercise training caused a significant reduction in Bcl-2 level, and Bcl-2/Bax ratio compared to T group \((P < 0.05)\) (Fig. 6A). Bcl-2 mRNA expression was down-regulated in all groups in comparison with T group. This change was more in T+TMX+EX group (0.075 fold) than T+EX and T+TMX groups (0.021 and 0.339 fold respectively) \((P < 0.05)\) (Fig. 6B).
3.6. TPM1 and PDCD4 expression

TPM1 mRNA expression was up-regulated in T+EX and T+TMX groups ($P < 0.05$). This effect was higher in T+EX group (12.71 fold) than other groups (T+TMX; 4.31 fold and T+TMX+EX; 11.25 fold) (Fig. 7A). PDCD4 expression was also up-regulated after 5 weeks of exercise training with the highest value in T+TMX+EX group ($P < 0.05$) (Fig. 7B). To study expression of possible proteins of TPM1 and PDCD-4 mRNA, confirmatory western blotting was performed. As shown in Fig. 7C, relevance of changes in gene expression of TPM1 and PDCD-4 identified by qRT-PCR in T, T+TMX, T+EX and T+EX+TMX groups, was also ratified by western blotting. We confirmed that both exercise training and tamoxifen increased TPM1 and PDCD-4 expressions. TPM1 and PDCD-4 expressions were increased in T+EX, T+EX+TMX and T+TMX groups compared to T group ($P < 0.05$) (Fig. 7C).

4. Discussion

The first goal of this study was to investigate the effects of interval exercise training and tamoxifen on tumor growth in mice bearing breast cancer; their effects on estrogen/ER-α, NF-kβ, IL-6 and STAT3 as upstream of miR-21 as well as TPM1, PDCD4 and Bax/BCL2 as downstream of miR-21. We demonstrated that tumor size and body weight were significantly declined in exercise training group in comparison with tumor group. Exercise training and tamoxifen induced expression of PDCD4, TPM1 and Bax, but decreased serum E2 and tumor IL-6 levels. They also down-regulated the expression of ER-α, NF-kβ, STAT3, and Bcl-2 in mice with breast tumors. These descriptive findings point to the role of exercise training together tamoxifen and miR-21 in cancer therapy.
In the present study, we used an interval exercise training protocol with gradual escalated intensity and volume pattern. We observed that exercise training produced lower body weight compared to other groups. Patel et al (2004) reported that calorie restriction had beneficial effects on tumorigenesis (Patel et al., 2004). Colbert et al (2009) suggested that lowering calorie by increased energy expenditure did not have the same effect as decreasing energy intake on mammary tumor development (Colbert et al., 2009). Therefore, increased calorie expenditure could decrease body weight, and may affect the tumor growth. However, we didn’t measure calorie intake in the present study.

Here, we observed lower tumor growth in exercise training and tamoxifen groups than tumor group. We also found a significant decrease in serum E2 level after interval exercise training and tamoxifen administration. Reportedly, physical activity and exercise could reduce adipose tissue, and thus have the potential to decrease circulating estrogen level (Patel et al., 2005). Additionally, increased expression of ERα is associated with an increased risk of cancer cell proliferation (Steiner et al., 2013). Tamoxifen, as ERα antagonist, inhibits mitogenic action of E2 in ERα-positive breast cancers (Howell et al., 1995). In this study, we found that exercise training reduced the circulatory levels of E2 and the ERα mRNA expression in tumor tissues. Therefore, our results are supported by other studies which have shown exercise training inhibitory effects on tumor growth.

According to clinical and epidemiological studies, there is a strong association between inflammation and cancer (Balkwill and Mantovani, 2001). IL-6, as an inflammatory molecule, was up-regulated in ERα positive human breast cancer (Sullivan et al., 2009). In this study, we found an increased level of IL-6 in tumor group. IL-6 promotes tumor growth by up-regulating anti-apoptotic and angiogenic proteins in tumor
cells (Trikha et al., 2003). Therefore, IL-6 may stimulate proliferation of breast cancer cells through estrogen production (Honma et al., 2002). Inflammatory responses can also lead to epigenetic switch between non-transformed and transformed cells that are mediated by a positive feedback loop involving NF-κB and IL6 as well as STAT3 (Frank, 2007; Yu et al., 2009). In this regard, IL-6 and NF-kB expressions were significantly decreased in animals with exercise training in the present study. Since estrogen can activate NF-κB via ERα (Mattson, 2008), and this in turn increases IL-6, hence lowered level of estrogen after exercise training may partly be responsible for reducing tumor burden. We also showed down-regulation of STAT3 expression after interval exercise training and tamoxifen administration. STAT3 is a factor capable of up-regulating miR-21 expression (Loffler et al., 2007). Moreover, IL-6 induced miR-21 expression in a STAT3-dependent manner (Loffler et al., 2007). Our results are in agreement with Iliopoulos et al. (2010) that STAT3 was directly activated miR-21 transcription during transformation process (Iliopoulos et al., 2010). In addition, Saxena et al. (2012) found that exercise training decreased STAT3 and IL-6 in mice treated with DSS-induced colitis (Saxena et al., 2012). We also showed down-regulation of miR-21 expression after interval exercise training and tamoxifen administration. High level of miR-21 expression is involved in proliferation, apoptosis, and migration of several cancer cell lines (Asangani et al., 2007; Zhu et al., 2008). MiR-21 is an anti-apoptotic and anti-inflammatory micro-RNA, and its high level in athletes is considered as a defective mechanism against apoptosis and inflammation (Urbich et al., 2008). The positive correlation of serum levels of miR-21 and IL-6 indicates the association between miR-21 and inflammation (Roy and Sen, 2011). There is a link between low maximal oxygen
consumption and high levels of circulating miR-21 expression in women (Bye et al., 2013). Endurance exercise training can decrease miR-21 level in circulation where acute exercise has no effect (Nielsen et al., 2014). Baggish et al. (2011) indicated miR-21 level was significantly decreased after exercise (Baggish et al., 2011). Therefore, exercise training may play an important role in miR-21 suppression in tumor tissue by down-regulating IL-6.

Many genetic alterations observed in breast cancer lead to an imbalance in the pro- and anti-apoptotic members of the Bcl-2 family. Overexpression of Bax promotes cell death, but Bcl-2 acts as a suppressor of apoptosis. As seen in exercise training, a decrease in Bcl-2/Bax ratio has been considered as a reliable indicator of the overall propensity of a cell to undergo apoptosis and target for anticancer therapy (Baell and Huang, 2002). It has generally been established that Bcl-2 mRNA expression was decreased in anti-miR-21-treated cells, miR-21 may indirectly regulate Bcl-2 expression (Yan et al., 2011).

Accordingly, one possible explanation would be the suppressive gene expression effects of anti-miR-21 with subsequent negative regulation of Bcl-2 expression. STAT3 could also modulate the expression of a number of anti-apoptotic proteins on the transcriptional level, such as Bcl-2 (Isomoto et al., 2005). Therefore, the effect of STAT3 on miR-21 may have an important role in controlling Bcl-2 expression. Si et al. (2006) also showed that inhibition of tumor growth was associated with increased apoptosis and decreased cell proliferation, which could be in part owing to miR-21 induced down-regulation of anti-apoptotic Bcl-2 (Si et al., 2006). In the present study, exercise training and tamoxifen increased Bax protein in animal tumor tissues. Estrogen can inhibit apoptosis via dual responses i.e., both stimulation of Bcl-2 and/or suppression of Bax productions (Wang et
al., 1995). These findings can reveal a new therapeutic potential for exercise training on miR-21 suppression, up-regulation of tumor suppressor such as PDCD4, TPM1 and apoptosis induction in mouse bearing mammary tumor.

Apoptosis role in breast carcinogenesis has extensively been studied, suggesting that resistance to apoptosis in premalignant breast epithelial cells will lead to the development of breast tumors (Mommers et al., 1999). In order to gain insight into mechanisms involved in apoptosis induction mediated by exercise training together with miR-21 inhibition of breast cancer development, we studied the effects of exercise training on the proteins levels of PDCD4, TPM1, STAT3, Bcl-2 and Bax under in vivo condition. PDCD4 is a novel tumor suppressor that is frequently down-regulated in breast cancer (Wang et al., 2008). It is also a cellular transformation inhibitor in mouse cell culture model. We found that PDCD4 mRNA expression is up-regulated in exercise training and tamoxifen treatment. Frankel et al. (2008) showed miR-21 role in PDCD4 regulation (Frankel et al., 2008). Moreover, Chang et al. (2011) demonstrated an inverse relationship between miR-21 and PDCD4 (Chang et al., 2011). According to Asangani et al. (2008), miR-21 post-transcriptionally down-regulates PDCD4 and stimulates tumor invasion (Asangani et al., 2007). TPM1 regulates anchorage-independent growth, and therefore is vital for transformation and cell invasion (Zhu et al., 2008). MiR-21 can enhance tumorigenesis and tumor growth by suppressing anti-growth factors including TPM1 (Zhu et al., 2007). Therefore, based on the presented findings, unlike mir21, TPM1 and PDCD4 expression were increased by exercise training in our study. These results may be due to down regulation of miR-21 and up regulation of its targets such as TPM1 and PDCD4.
5. Conclusion

The exercise training has inhibitory effects on tumor growth in different animal models of cancer (Mehl et al., 2005; Murphy et al., 2011), however, its mechanistic effects remains unclear. MiR-21 as oncogene was over-expressed in solid tumors such as estrogen receptor (ER)-positive breast cancer (Iorio et al., 2005). It can be a predictor in cancer prognosis, and has a potential to be used as an indicator of therapeutic outcome in many cancerous states such as breast malignancies (Yan et al., 2008). The present study showed that the exercise training plus tamoxifen can inhibit tumor growth. Five weeks of interval exercise training and also tamoxifen administration could down-regulate the ER-α, NFk-B and STAT3, and subsequently down-regulate miR-21. These effects could induce the apoptotic factor such as Bax, and inhibit the Bcl-2 as anti-apoptotic mediator. Tamoxifen together with exercise training augmented tumor growth suppression. We can conceivably conclude that interval exercise training may act as an adjuvant to hormone therapy in breast tumors and should be considered for further investigation in cancer arena for its potential helpful effects.

Declaration of interest

The author(s) report no conflicts of interest and are responsible for the content of the paper.

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References


Legends

Fig. 1. Illustration of the experimental protocols

Forty-eight mice were equally divided into six groups (I) control (C); healthy animals with neither tumor induction nor exercise training protocol, (II) exercise training (EX); animals under exercise training protocol for 5 weeks without tumor cell injection, (III) tumor induction (T); animals with breast tumor, (IV) tumor+exercise (T+EX); animals with breast tumor underwent 5 weeks of exercise training protocol after tumor establishment, (V) tumor+tamoxifen (T+TMX); TMX (5 mg/kg, daily) was given via oral gavage for 2 weeks after tumor establishment, and (VI) tumor+tamoxifen+exercise (T+TMX+EX).

Fig. 2. Effects of exercise training and tamoxifen on (A) body weight (g) and (B) tumor size (cm$^3$) in mammary tumor-bearing mice

Tumor volume was measured by a digital vernier caliper on a weekly basis and reported as cm$^3$ using the following formula: $V = \frac{1}{6} (\pi LWD)$, where $L =$ length, $W =$ width and $D =$ depth. Data reported are mean ± S.D. *$P < 0.05$ compared to tumor group. C: control,
EX: exercise, T: tumor, T+EX: tumor+exercise, T+TMX: tumor+tamoxifen and
T+TMX+EX: tumor+tamoxifen+exercise.

**Fig. 3.** Effects of exercise training and tamoxifen on (A) serum 17β-estradiol levels and
(B) ER-α mRNA expression in mammary tumor-bearing mice.

Data reported are mean ± S.D. *P < 0.05 compared to tumor group, #P < 0.05 compared
to control group. C: control, EX: exercise, T: tumor, T+EX: tumor+exercise, T+TMX:
tumor+tamoxifen and T+TMX+EX: tumor+tamoxifen+exercise.

**Fig. 4.** Effects of exercise training and tamoxifen on (A) IL-6 levels, (B) NF-kB mRNA
levels and (C) STAT3 mRNA levels in mammary tumor-bearing mice.

Data reported are mean ± S.D. *P < 0.05 compared to tumor group, EX: exercise, T:
tumor, T+EX: tumor+exercise, T+TMX: tumor+tamoxifen and T+TMX+EX:
tumor+tamoxifen+exercise.

**Fig. 5.** Effects of exercise training and tamoxifen on tumor tissue miR-21 expression in
mammary tumor-bearing mice.

Data reported are mean ± S.D. *P < 0.05 compared to tumor group, EX: exercise, T:
tumor, T+EX: tumor+exercise, T+TMX: tumor+tamoxifen and T+TMX+EX:
tumor+tamoxifen+exercise.

**Fig. 6.** Effects of exercise training and tamoxifen on (A) Bax and Bcl-2 proteins, and (B)
BCL-2 mRNA level in tumor tissue of mammary tumor-bearing mice.

Data reported are mean ± S.D. *P < 0.05 compared to tumor group, EX: exercise, T:
tumor, T+EX: tumor+exercise, T+TMX: tumor+tamoxifen and T+TMX+EX:
tumor+tamoxifen+exercise.
Fig. 7. Effects of exercise training and tamoxifen on (A) TPM1 mRNA level, (B) PDCD4 mRNA level, and (C) expression of PDCD4 and TPM1 proteins in mammary tumor-bearing mice

Data reported are mean ± S.D. *P < 0.05 compared to tumor group, EX: exercise, T: tumor, T+EX: tumor+exercise, T+TMX: tumor+tamoxifen and T+TMX+EX: tumor+tamoxifen+exercise.

Table 1. PCR primers for miR-21, PDCD4, BCL2, STAT3, TPM1, NF-kB, and ER-α

<table>
<thead>
<tr>
<th>Gens</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>NCBI</th>
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<tr>
<td>MiR-21</td>
<td>UAGCUUAUCAGACUGAUGUGUA</td>
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<td>NR_029738</td>
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<td>U6</td>
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<td>GTGCAGGGTCCGAGGT</td>
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<td>NF-KB</td>
<td>GAAATTCCTGTATCCAGACAAAAAC</td>
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<td>STAT3</td>
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<td>BCL2</td>
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<td>PDCD4</td>
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<td>TPM1</td>
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<td>ER-α</td>
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<td>GAPDH</td>
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<td>ACCCTGGTGTGCTGCACGT</td>
<td>NM_008084</td>
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</table>
Figure 1

I  
Healthy Animals

II  
Exercise Training

III  
Tumor establishment
Exercise Training

IV  
Tumor establishment
Exercise Training

V  
TMX (5 mg/kg, daily)
Euthanasia

VI  
Tumor establishment  Time (Weeks)
Figure 3

A

17β-estradiol serum levels (pg/ml)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Exercise: P</th>
<th>Tamoxifen: P</th>
<th>Interaction: P</th>
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<tr>
<td>C</td>
<td>0.0001</td>
<td>0.167</td>
<td>0.0001</td>
</tr>
<tr>
<td>EX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T+EX</td>
<td>*</td>
<td></td>
<td>#</td>
</tr>
<tr>
<td>T+TMX</td>
<td></td>
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</tr>
<tr>
<td>T+TMX+EX</td>
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<td></td>
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</table>

B

ER mRNA (fold changes to control)

<table>
<thead>
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<th>Condition</th>
<th>Exercise: P</th>
<th>Tamoxifen: P</th>
<th>Interaction: P</th>
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<tbody>
<tr>
<td>T</td>
<td></td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>T+EX</td>
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<tr>
<td>T+TMX</td>
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</tr>
<tr>
<td>T+TMX+EX</td>
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</tr>
</tbody>
</table>
Figure 4

A

Exercise: P = 0.0001
Tamoxifen: P = 0.001
Interaction: P = 0.013

IL-6 Levels in Tumor (pg/mg)

T | T+EX | T+TMX | T+TMX+EX

B

Exercise: P = 0.001
Tamoxifen: P = 0.0001
Interaction: P = 0.001

NF-κB mRNA (Fold changes to control)

T | T+EX | T+TMX | T+TMX+EX

C

Exercise: P = 0.003
Tamoxifen: P = 0.027
Interaction: P = 0.041

STAT1 mRNA (Fold changes to control)

T | T+EX | T+TMX | T+TMX+EX
Figure 5

Exercise: $P = 0.0001$
Tamoxifen: $P = 0.002$
Interaction: $P = 0.013$
Figure 6

(A) Bar chart showing the mean expression of Bax and Bcl-2 with different conditions:
- **T**: Baseline
- **T+EX**: Exercise
- **T+TMX**: Tamoxifen
- **T+TMX+EX**: Exercise + Tamoxifen

- **Bax** (black bars) and **Bcl-2** (grey bars) expression levels are indicated.

(B) Bar chart showing Bcl-2 mRNA fold changes to control:
- **T**: Baseline
- **T+EX**: Exercise
- **T+TMX**: Tamoxifen
- **T+TMX+EX**: Exercise + Tamoxifen

- **Exercise**: $P = 0.001$
- **Tamoxifen**: $P = 0.067$
- **Interaction**: $P = 0.236$
Figure 7

A

Exercise: \( P = 0.0001 \)
Tamoxifen: \( P = 0.99 \)
Interaction: \( P = 0.61 \)

B

Exercise: \( P = 0.001 \)
Tamoxifen: \( P = 0.041 \)
Interaction: \( P = 0.833 \)

C

TPM1  
PCDC4  
\( \beta \)-actin