Original Article

Investigation of the Effect of High-Intensity Interval Training (HIIT) on the Expression of the Genes miR-126, miR-296, HGS and VEGF-A Protein Levels in Tumor Tissue in Female Mice

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Abstract

Background & Objective: The studies of the last two decades have shown that regular training is associated with the reduction of mortality among patients with breast cancer, and has an important role in inhibiting breast cancer progression as well as treatment of the disease. However, the micro-molecular mechanisms involved are not fully understood yet. However, the aim of the present study was to investigate the effect of a high-intensity interval training (HIIT) on the expression of miR-126, miR-296, HGS gene, and VEGF-A protein level in tumor tissue of the mice with breast cancer.

Materials & Methods: For this purpose, 12 BALB/c mice (6-8 weeks, weight 19 ± 1.05 g), after induction of cancer (MC4-L2 subcutaneous injection into the right side of the mice), were randomly divided into two groups with 6 in each group: HIIT and control. Each session of HIIT group program includes six intervals of three minutes and 20 seconds with an intensity of 85 to 90 percent of VO2max and 1-minute recovery with 30 to 35 percent VO2max intensity between each interval; and the animals were sacrificed 24 hours after the last training session and the expression of miR-126, miR-296, HBS gene and VEGF protein levels in tumor tissues were examined.

Results: The statistical results showed that the implementation of HIIT significantly reduced the expression of miR-296 and subsequently increased the HGS gene expression and led to increased expression levels of miR-126 and decreased levels of VEGF-A protein in the training group compared to the control group ($p<0.05$).

Conclusion: It seems that HIIT, by suppressing tumor angiogenesis signaling pathways, could be an effective intervention to inhibit the growth of breast tumors.

Keywords: Breast Cancer, Exercise training, miR-126, miR-296, VEGF-A

Introduction

Breast cancer is one of the most common types of cancer among women and is considered one of the most common causes of death around the world which has rapidly growing financial costs and clinical complications (1). For decades, many researches have been done at the molecular level to clarify the molecular mechanisms involved in the development and progression of tumor. Because of the unknown mechanism of the cancer, no definite treatment has been presented yet. On the other hand, there are various presumptive mechanisms which have been considered for this illness the most common of which is angiogenesis. Cancer progression is dependent on the formation of a new system of vessels (angiogenesis) (2) and this process is highly dependent on family of vascular endothelial growth factor (VEGF) and their receptors (VEGFR). Angiogenesis mechanism

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leads to the formation and expansion of new tumor blood vessels, tissue invasion and metastasis and tumor growth (3). In addition, in the last decade, researchers discovered a group of non-coding RNAs called microRNAs that are considered as biomarkers for early diagnosis and as therapeutic agents (4). MiRNAs are a large group of non-coding RNAs that play a key role as oncogenes and tumor suppressor genes, angiogenesis, tissue invasion and metastasis (5). MiR-126 is one of these MiRNAs which is expressed specifically in endothelial cells and its expression can lead to the induction of angiogenesis in tumor(6,7). MiR-126 which significantly decreases in breast tumor tissue (8,9) is associated with targeting multiple genes including VEGF-A, and increased expression of these genes and activation of the VEGF / PI3K / AKT signaling pathway which eventually leads to tumorgenesis and tumor growth (9). The degradation of VEGF receptors is mediated by hepatocyte growth factor-regulated tyrosine kinase substrate (HGS). MiR-296 inhibits HGS, stimulating growth-factor mediated angiogenesis(10).

HGS gene is one of the regulatory factors of angiogenesis pathway in vascular endothelial cells. It causes a decline in angiogenesis of cancerous tumors and prevents tumor growth by downregulating angiogenic growth factor receptors, such as VEGFR2 and PDGFRB(6).

The other MiRNAs, increased expression of miR-296 in tumor tissue by targeting HGS gene and connection to area 3'UTR of the gene and its expression reduction, leads to increased proangiogenetic endothelial growth factor receptors such as VEGFR2 and PDGFRB in tumor tissue (11).

It seems that non-pharmaceutical intervention is an important and necessary option in controlling the disease. In recent years, evidence has shown that exercise training reduces breast cancer risk factors, including sexual hormones, insulin levels, and inflammatory markers, and adipose tissue, and results in decreased risk of breast cancer among women (12,13).

In recent decades, studies have shown that regular physical activity is associated with reduced mortality in patients with cancer, although the molecular mechanisms involved in the relationship are still not fully understood (14-16). Reducing the metastatic spread of tumor cells and inhibiting the growth of breast tumors, physical activities have an important role in controlling the disease (17-19).

Recent observational evidence suggests that moderate levels of physical activity may even reduce the risk of death from breast cancer, and therefore exercise may prove to be a valuable intervention to improve not only quality of life but overall survival. It is also associated with improving physical function and reducing fatigue caused by cancer, during treatment period and after that. Therefore, the unique characteristics of physical activity have made it an effective non-pharmacological intervention to control and prevent breast cancer (20,21). While there is strong evidence to support that exercise interventions improve breast cancer-related factors compared to a sedentary control group or standard care, comparatively little information is available to examine which frequency, intensity, time, or type of exercise is best (22-24). Associations of Sports Medicine have recommended 150 min/wk (30 min, 5 d/wk) of moderate intensity aerobic exercise for patients with breast cancer (21). Lynch et al. analyzed more than 70 studies in relation to physical activity and breast cancer. Accordingly, they reported that high-intensity training is more effective in reducing the risk of breast cancer (13). The evidence also shows that high-intensity training is likely better and more effective in controlling the markers related to reduction of risk of breast cancer (25,26). Accordingly, it seems that High-Intensity Interval Training (HIIT) may also be able to reduce complications of breast cancer effectively. HIIT protocol includes trainings with intense repeated periods and relatively short with rest periods between repetitions (27). Given the short time allocated, this training model can be more compliments by patients with breast cancer. Therefore, further study is needed to determine the true role of tumor angiogenic factors and the effect of HIIT on inhibiting breast cancer progression. Previous interventions may not be sufficient, average, or sufficient to stimulate significant and sustained elevations in angiogenic tumor factors.

Materials & Methods

Animals

The present study was performed on 12 BALB/c mice (6-8 weeks, 19 ± 1.05 g weight) prepared from the Pasteur Institute of Iran. Animals were kept in standard laboratory

conditions (23-25 ° C, 40-50% humidity, the radiation-darkness cycles of 12:12), and they freely had access to food and water for laboratory animals. All the ethics of the study were observed according to the principles of working with laboratory animals adopted by Tehran University of Medical Sciences. In addition, the code of ethics for the above study is IR.IAU.M.REC.1399.001.

Cell culture

The MC4-L2 cells that are Estrogen Receptor-positive (ER+) breast ductal carcinoma were purchased from Iran Genetic Resources Center. MC4L2 cells were cultured in T75 flask in DMEM/F-12 environment with 15 mmol HEPES buffer, glutamine, 100 mg/ml penicillin, 100 mg/ml streptomycin and 10% FBS.

Tumor induction

After culturing MC4-L2 cells in culture environment and preparation of cell suspension, one million suspension cells in PBS were injected subcutaneously into the right side. Then, after the appearance of breast tumors, all mice were randomly divided into two groups of six: intensive control and interval groups.

Exercise protocol

Each HIIT protocol session consisted of 35 minutes of running on the treadmill, so that rats warmed up for five minutes at first running with intensity of 30-40% VO2max. Then, six intervals were performed (each interval lasted for 20 seconds running with the intensity of 85- 90 % VO2max and one minute recovery with the intensity of 30-35 % VO2max between each interval) and at the end running with the intensity of 30-40% VO2max for five minutes to cool down.

Assessment of tumor volume

At the end of each week, tumor volume was bi-dimensionally measured using a digital caliper. The longer dimension was measured as the length of the tumor while the other dimension (at an angle of 90°) was measured as width. Tumor volume was calculated as $\pi/6 \times \text{width} \times \text{length}^2$, which is a standard formula for calculating tumor volume in mouse models of breast cancer.

Necropsy

24 hours after the last training session, the mice were anesthetized by intraperitoneal injection of ketamine and xylazine, 100 and 10 mg.kg-1, respectively(28). Then, the tumor tissue was extracted and immediately frozen at -80 ° C nitrogen and stored for analysis.

MiRNA expression by Real-Time-PCR

Total RNA and small RNA from tumor tissue were extracted using RNeasy Mini Kit (Qiagen, Germany), and total RNA (1 mg) and small RNA (2 mg) were first reversely transcribed into cDNA using miScript II RT Kit, respectively. Quantitative real-time polymerase chain reaction (PCR) (qPCR) was performed with SYBR Green RT-PCR Master Mix kit (Qiagen, Germany) in a Rotrogene 6000 system (Corbet, Germany) using the miR-126 and miR-296 primers set and SNORD-61 primers set (Qiagen, Germany). All samples were normalized to internal controls, and the relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ analysis method. Experiments were performed in duplicate samples.

MRNA expression by Real-Time-PCR

Total RNA and small RNA from tumor tissue were extracted using RNeasy Mini Kit (Qiagen, Germany), and total RNA (1 mg) and small RNA (2 mg) were first reversely transcribed into cDNA using Transcriptor first strand cDNA synthesis kit (Roche, Germany), respectively. Quantitative real-time PCR (qPCR) was performed with SYBR Green RT-PCR Master Mix kit (ampliqon, Denmark) in a Rotrogene 6000 system (Corbet, Germany) using HGS primers set and GAPDH primers set (Sina gene, Iran). All samples were normalized to internal controls, and the relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ analysis method. Experiments were performed in duplicate samples.

Western Blot Analysis

For Western blot analysis, tissue samples were centrifuged and re-suspended in ice cold RIPA lysis buffer (0.5 M Tris–HCl, pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA) in the presence of Protease Inhibitor Cocktail. To confirm the expression of VEGF-A, 30 µg of protein was loaded and size-fractionated on a 10% sodium dodecyl sulphate-polyacramide gel electrophoresis (SDS–PAGE) and blotted overnight onto a PVDF membrane. The membranes were blocked with TBS-T (Tris-buffered saline containing 0.3% Tween 20) and 5% nonfat dry milk for 1 h at room temperature. Western blotting was done using antibodies against VEGF-A (Abcam, Cambridge, MA, USA) and β-actin (Abcam, Cambridge, MA, USA) at 4ºC. The band density was normalized using the Image J software.

Data analysis

All the data were reported as mean ± standard deviation. Statistical comparisons were performed between the two groups using t-test. The data were analyzed by statistical package for social sciences (SPSS) 19, at significance level of $p<0.05$. To quantify Western-blot images and to draw charts, Graph Pad *Prism* was used.

Results

HIIT reduces tumor growth

The data from the tumor volume in the study showed that the implementation of HIIT results in the inhibition of tumor growth in the training group compared to the control group so that tumor volume in HIIT group in the tenth week had a growth of 8.47 fold greater than the first week, and in the control group, tumor volume growth was 12.13 fold greater than the first week (Table 1and 2). Also, in Table 3, miR-296, miR-126 and HGS gene expression values and VEGF-A protein in the research groups are presented.

HIIT inhibits the VEGF/VEGFR signaling pathway by Regulation of miR-126 and miR-296

MiR-126 expression level in tumor tissue of HIIT group was significantly higher compared with the control group ($p<0.05$) (Chart. 1A), and the results of Western blot showed decreased levels of VEGF-A protein in the HIIT group compared to the control group ($p<0.05$) (Chart. 2). In addition, our data also showed that the implementation of HIIT leads to a significant decrease in the expression level of miR-296 in the training group compared to the control group ($p<0.05$) (Chart. 1B). The results also

Table 1. Mean tumor volume in different groups (tumor volume per cubic millimeter)

Group week	H	C
first	177.44±49.25	108.38±24.39
tenth	1503.96± 242.50	1314.90± 125.27

The data presented as mean ± SD

Table 2. The mean Tumor Volume

Variable	Control	HIIT
Tumor Volume mm^3	12.132	8.476*

*: Significant difference compared to control group.

Table 3. MiR-296, miR-126 and HGS gene expression and VEGF-A protein in research groups (Mean ±standard error)

Group Variable	C	H
miR-296	1	0.614± 0.42
MiR-126	1	1.431± 0.10
HGS	1	1.658± 0.45
VEGF-A	14.658± 1.229	6.9940± 1.116

indicated a significant increase in HGS gene expression in tumor tissue of HIIT group compared to the control group ($p<0.05$) (Chart. 1C).

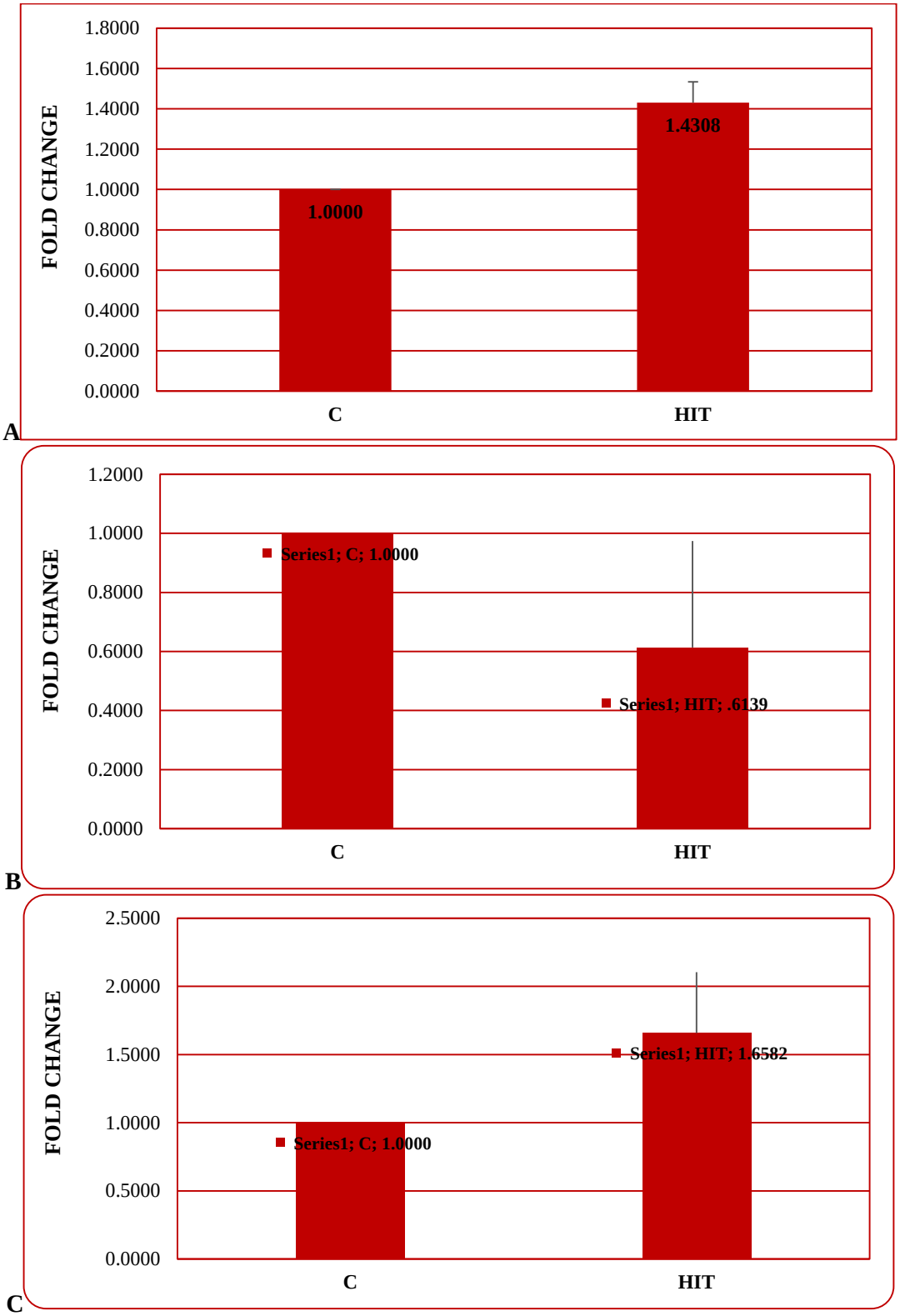

Chart 1. Effect of HIIT protocol on gene expression at mRNA level. (A) miR-126 expression, (B) miR-296 expression, (C) HGS expression. The data presented as mean ± standard deviation *: significant differences between HIIT and Control groups.

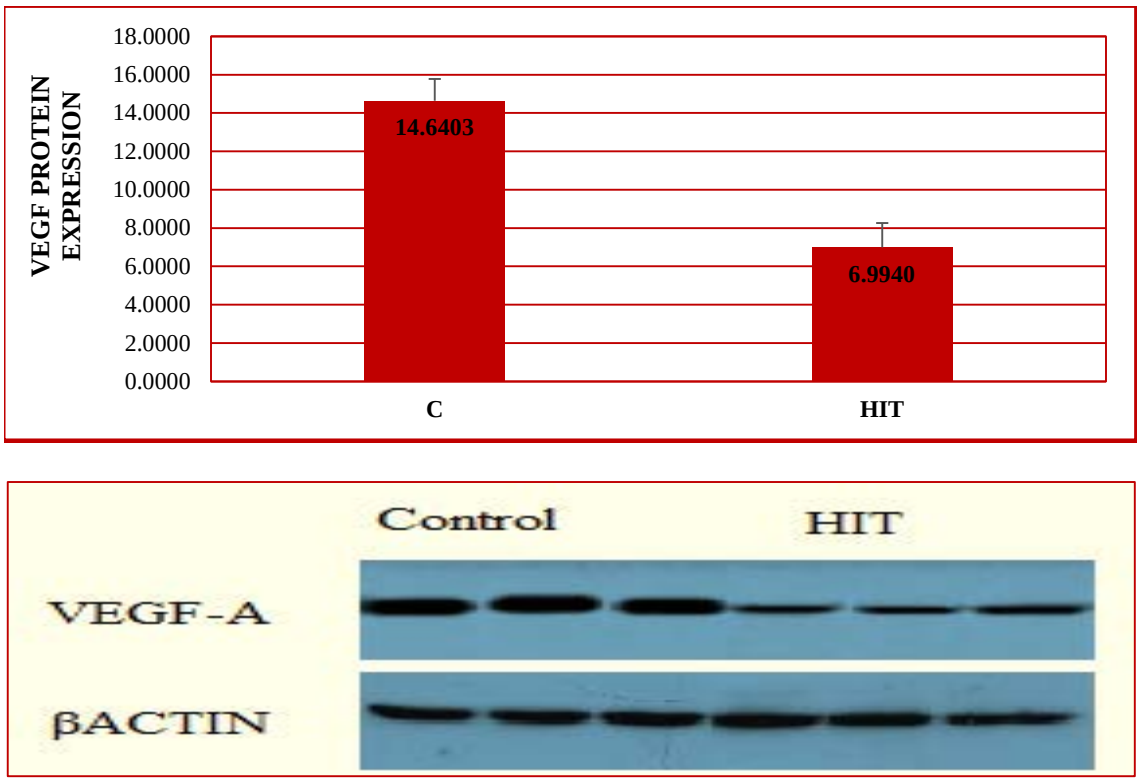

Chart 2. Effect of HIIT protocol on VEGF-A protein. VEGF-A. The data presented as mean ± standard deviation *: significant differences between HIIT and Control groups.

Discussion

Our research shows that the implementation of HIIT can significantly inhibit breast tumor growth so that in the HIIT group, tumor volume in the tenth week, had a growth of 8.47 fold greater than the first week, and in the control group, tumor volume growth was 12.13 fold greater than the first week. In this regard, Zielinski *et al.* reported that prolonged high-intensity training causes a delay in tumor growth, reduction in the number of inflammatory cells as well as a reduction in the number of tumor blood vessels (29). Moreover, Bacurau et al. showed that the implementation of HIIT leads to a significant reduction in tumor volume in tumor-bearing rats (30).

The nature of exercise training involves repetitive bouts of exercise that challenge whole-body homeostasis, leading to widespread adaptations in cells, tissues, and organ systems(31). Evidence is emerging from murine studies showing that exercise training 1) controls cancer progression through direct effects on tumor intrinsic factors (growth rate, metastasis, tumor metabolism, and immunogenicity of the tumor), 2) regulates tumor growth through interplay with systemic factors, 3) alleviates adverse events related to cancer and its treatment, and 4) improves cancer treatment efficacy (32). Intratumoral signaling networks are highly modifiable and modulated by numerous extrinsic factors (33). In the case of exercise, these extrinsic factors include both physical effects (i.e., increase in blood flow, shear stress on the vascular bed, pH regulation, heat production, and sympathetic activation) and endocrine effects (i.e., stress hormones, myokines, and circulating exosomes) (34), all of which have the potential to regulate cancer progression and biology. The effect of these physiological factors may regulate tumor growth kinetics, metastatic potential, tumor metabolism, and the immunogenic profile of the tumor. Exposure to exercise-induced

molecular factors may interfere with molecular signaling events in the cancer cells that are involved in tumor formation. One such pathway, which is essential for organ formation and has been implicated in tumor formation, is the Hippo signaling pathway (35). Recent studies have demonstrated that this pathway is downregulated by exercise and catecholamines (36). In addition, exercise and exercise-conditioned serum have been shown to deactivate Hippo/YAP signaling in breast cancer cells through an epinephrine-dependent mechanism, and blockade of adrenergic signaling blunted the suppressive effect of exercise-conditioned serum on both tumor formation and cell viability (37). Studies indicate that events triggering immune infiltration, and partly alleviating immunosuppressive metabolites, may act to promote enhanced immunogenicity within tumors from exercising mice(32).

In estrogen receptor dependent breast cancer, estrogen can activate NF-κB when it binds to its receptor. It leads to increase in IL-6 levels, which results in the suppression of programmed cell death 4 (PDCD4) and tropomyosin 1 (TPM 1) and up-regulation of B-cell lymphoma protein (BCL2), in addition to the stimulation of induction VEGF production for angiogenesis, through IL-6/STAT3 pathway(38).

High levels of VEGF in primary tumors with the induction of angiogenesis lead to increased metastases and more growth of breast tumor. Thus, the inhibition of signaling pathway of VEGF/VEGFR now is one of the researchers' therapeutic targets to inhibit tumor angiogenesis (3,39). Angiogenesis is a main characteristic of cancer progression which has long been considered as a purpose of treatment. Because VEGF is considered as the most important factor for angiogenesis and is directly associated with metastasis and tumor growth, it can serve as a therapeutic aim in suppressing tumor angiogenesis-induced growth (40). In this regard, Fokens et al. demonstrated that high amounts of VEGF in the primary tumor are associated with increase in metastasis and further growth of tumor in patients with breast cancer (41). Besides that, microRNAs are the regulators of gene expression at post-transcription level, which have recently attracted much attention because of their major contributions as oncogenes or tumor-suppressing genes, to the development of cancers, as well as their important roles in oncogenesis and metastasis (42).

Our data also showed that the expression of miR-126 in HIIT group was significantly higher compared with the control group. It seems that the expression of miR-126 resulted in decreased expression of VEGF-A protein at the protein level so that the amount of this protein decreased in HIIT group. In line with these results, Verma *et al.* reported that regular physical activity in tumor-bearing mice resulted in reduction of VEGF expression in tumor tissue, as well as reduction in tumor volume (43). Zhu *et al.* also observed that aerobic activity of moderate intensity leads to decreased expression of VEGF and vascular density in the breast tumor of rats (44). Therefore, it can be said that the significant increase in levels of miR-126 and the subsequent reduction of VEGF-A protein in tumor tissue of the training group, can be one of the reasons for the decreased tumor growth.

High levels of proangiogenic VEGFRs such as PDGFR-β and VEGFR2 is one of the outstanding characteristics of angiogenic blood vessels. On the other hand, by targeting HGS gene and decreasing its expression, miR-296 results in increased VEGFRs (PDGFR-β, VEGFR2) (12). Our findings showed that expression of miR-296 in HIIT group was lower compared to the control group. This decreased expression of miR-296 in HIIT group led to a significant increase in HGS gene expression. In this regard, Wurdinger *et al.* demonstrated that the inhibition of miR-296 by antagomirs leads to increased HGS gene expression and subsequently, decreased angiogenesis in tumor tissue (12). So, it seems that the

significant reduction in miR-296 levels in tumor tissue of HIIT group is probably one of the reasons for the decreased tumor growth.

Conclusion

The results of the present study show that by adjusting antiangiogenic and proangiogenic factors, HIIT suppresses signaling pathways of angiogenesis tumor. Since the formation of new vasculature system and angiogenesis is necessary for metastasis and tumor growth, the implementation of HIIT can have effective intervention to inhibit tumor growth.

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Conflicts of Interest

The authors declare no conflict of interest.

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