The Effect of Exercise on Gene Expression and Signaling in Mouse Melanoma Tumors

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1Exercise Physiology and Biochemistry Laboratory, College of Physical Education, Jinggangshan University, Ji’an, CHINA; 2School of Sports Medicine and Health, Chengdu Sport University, Chengdu, CHINA; 3Department of Kinesiology, Coastal Carolina University, Conway, SC; 4Department of Physical Education, Federal University of Maranhão (UFMA), São Luis-MA, BRAZIL; and 5Laboratory of Cellular and Molecular Biology of Skeletal Muscle (LABCEMME), BRAZIL

ABSTRACT

XIA, Z., H. SHANG, J. CHOLEWA, Q. WANG, X. DING, Q. SU, Y. ZHAO, and N. E. ZANCHI. The Effect of Exercise on Gene Expression and Signaling in Mouse Melanoma Tumors. Med. Sci. Sports Exerc., Vol. 52, No. 7, pp. 1485–1494, 2020. Purpose: To screen for candidate hub genes associated with the effects of exercise on melanoma tumor tissues and to review the potential signaling pathways involved in this process using bioinformatics analysis. Methods: The GSE62628 expression profile was downloaded from Gene Expression Omnibus database. This data set contains 10 melanoma tumor tissues from two groups of exercise and nonexercise mice. The R software was utilized to identify differentially expressed genes between samples, and functional annotation and pathway analysis were performed. Results were visualized using Cytoscape software. Results: In total, 315 differentially expressed genes were obtained, including 294 upregulated and 21 downregulated genes. The functional analysis showed that these genes were mainly enriched in immune response, inflammatory response, and positive regulation of the ERK1/2 cascade in biological process functional groups. The top 10 candidate hub genes were C3, Kng1, C3ar1, Ptafr, Fgg, Alb, Pf4, Orn1, Aldh3b1, and Apob. The pathway analysis of the most significant module identified from the protein–protein interaction network revealed that the complement and coagulation cascades, Staphylococcus aureus infection, cytokine–cytokine receptor interaction, chemokine signaling pathway, and phagosome were mainly involved. C3, C3ar1, Kng1, Ptafr, and Fgg may be the critical genes in the complement and coagulation cascades pathway, and S. aureus in the infection pathway. Conclusions: Exercise may ameliorate the immune response and inflammatory response in melanoma tissue, and further studies exploring their relationships are warranted. Key Words: EXERCISE, MELANOMA, GEO DATABASE, BIOINFORMATICS ANALYSIS, DIFFERENTIALLY EXPRESSED GENES, BIOLOGICAL PATHWAYS

Cancer is ranked as one of the leading causes of death worldwide, resulting in a heavy social and economic burden on the public health sector. A high volume of research has been conducted to explore potential strategies to prevent and/or treat cancer and its secondary harmful influence (1), with an increasing emphasis on the preventative effects of exercise (2–5). According to the continuous update project expert report of 2018 released by World Cancer Research Fund/American Institute for Cancer Research, all cancer survivors should perform at least moderate physical activity (6). However, there is limited data on the effects of physical activity and exercise on prognosis and quality of life during and posttreatment. There are clear differences between the effects of exercise on cancer prevention, versus treatment and survival, and exercise would never be prescribed for most cancers without traditional treatment. Moreover, there is no evidence that exercise in itself can directly eradicate a tumor. Thus, exercise is more like an adjunct therapy along with traditional clinical treatments, and a better understanding of the underlying biological mechanisms linking physical activity and exercise to cancer prevention and outcomes in cancer survivors is warranted.

Melanoma is the deadliest and most metastatic form of skin cancer which affects tens of thousands of people worldwide annually, and the prevalence is growing faster than any other type of solid cancer (7). The risk factors for melanoma are well known, including both intrinsic and extrinsic factors. Except for variances in genotype and phenotype, the most important extrinsic cause is sun exposure. Exercise may help reduce the risk of skin cancer, however, the current state of the literature is ambiguous. For example, Moore et al. found that increased levels of physical activity in leisure time were associated with an increased risk of melanoma (8), while a case-control study found that higher levels of physical activity
are associated with a 30% reduction in melanoma risk (9). Physical activity and exercise are often done in thin clothing and outdoors, which poses an increased risk of sun exposure and sunburns. In addition, Moore et al. found that exercise was more associated with melanoma in areas with high ultraviolet radiation, and suggested that solar irradiance was an important trigger for this relationship. It is currently unclear whether exercise protects against melanoma, and if so, via which mechanisms.

During the last decade, microarray technology and bioinformatics analysis have been widely used to screen genetic alterations at the genome level, which have helped to identify differentially expressed genes (DEGs) and signaling pathways involved in the carcinogenesis and progression in individual cancers (10). The bioinformatics strategy is an uncommon analysis in the field of sports medicine, and original data sets on this topic in the public databases are scarce. Thus, this is the first research to our knowledge to investigate the potential targets and signalings of voluntary exercise on melanoma tumor tissues via the bioinformatics strategy. Recently, Wang et al. used gene profile analysis to compare 2 sets of subcutaneous white adipose tissue samples in C57BL/6 mice to discover DEGs of sedentary and exercise interventions. SLC27A1, COX7A1, PPARC1A, FABP3, and UCP1 were identified as top 5 hub genes in white adipose tissue after 11 d of exercise, and the PPAR signaling pathway, adipocytokine signaling pathway, and the insulin resistance pathway were screened out as the critical pathways involved in the enhanced relationship between the browning of white adipose tissue and insulin resistance by exercise (11). Similar analyses in the oncology research field can also be found in the works of Li et al (10), Trigos et al (12), and Vidotto et al (13) as examples.

To our knowledge, the effects and potential mechanisms of exercise on melanoma tumors require further study, and there is no consensus among researchers regarding these issues. Recently, a preclinical study by Pedersen et al (14) investigated the effects of 6 wk voluntary wheel running on the melanoma tumor tissues, using B16F10, a fast-growing transplantable tumor model. Exercise was found to decrease melanoma tumor incidence and growth by over 60% through the regulation of epinephrine- and IL-6-dependent manner. In the present study, we performed bioinformatics analysis based on Pedersen et al.’s data set (GSE62628) to further compare those 2 sets of melanoma tumor samples to screen for DEGs of sedentary and exercise interventions. We aimed to identify the potential hub genes and signaling pathways using a protein–protein interaction (PPI) network to predict a potential mechanic model of exercise.

METHODS

Animals and exercise intervention from the original experiment. In the original melanoma experiment conducted by Pedersen et al., 3-month-old female mice were inoculated subcutaneously with \( 2 \times 10^5 \) B16F10 melanoma cells. Two weeks after injections, the mice were sacrificed posteuthanization, and tissues of interest were excised. The melanoma tumors were dissected into fragments and incubated 4 h in a digestion enzyme mixture of Roswell Park Memorial Institute 1640 with 0.4 mg·mL\(^{-1}\) collagenase I and 10 U·mL\(^{-1}\) pullozyme. Digested tumor fragments were resuspended and filtered (70 μm cell strainer cap) and washed twice in phosphate buffer saline + 2% fetal bovine serum. Total RNA was extracted using the TRIzol reagent. In regard to the exercise intervention, a 12-cm-diameter running wheel was placed in the cage as a model of voluntary exercise. Access to running wheels was given 4 wk before tumor cell inoculation and during the 2-wk tumor challenge. Total running distance was evaluated by placement of bicycle computers on the running wheels, and the average wheel running distance was 4.1 km per mouse per day. The results showed that exercise before and during B16F10 tumor challenge reduced tumor growth and volume by 61% \((P < 0.01)\) and 67% \((P < 0.05)\), respectively (14).

Microarray data. The microarray gene profile (ID: GSE62628, published by Pedersen et al. [14]) of melanoma tumor tissue from two groups of exercise (n = 5) and nonexercise C57BL/6 mice (n = 5) was downloaded from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). Gene expression levels were measured using GPL6246 [MoGene-1_0-st] Affymetrix Mouse Gene 1.0 ST Array (Affymetrix Inc. Santa Clara, CA).

Microarray data preprocessing. The platform and raw expression data in Series Matrix format were downloaded as TXT files. The data were preprocessed by using the affy and preprocess Core package from Bioconductor (Bioconductor, Fred Hutchinson Cancer Research Center, Seattle, WA). In this process, data were calibrated, standardized, and log2 transformed. Thereafter, probe identifier (ID) sets were converted into gene symbol by using the Bioconductor annotation package mogene10stranscriptcluster.db in the microarray platform (15).

Differential expressed genes screening. To identify the DEGs between sedentary and exercised samples, limma package (http://www.bioconductor.org/packages/release/bioc/html/limma.html) of Bioconductor was used (16), which provided unpaired t-test to calculate the \( P \)-value for expression differences. DEGs with a corrected \( P \)-value < 0.05 and |Log Fold Change (FC)| ≥ 0.75 were considered as the significant DEGs.

Cluster analysis of differential expressed genes. Cluster analysis was performed using heatmap package in R. Euclidean distance was adopted to cluster the genes and produce the dendograms.

Functional enrichment analysis of differential expressed genes. To investigate the biological functions of DEGs, the online tool, Database for Annotation, Visualization and Integration Discovery (DAVID, http://david.abcc.ncifcrf.gov/) was utilized to perform the GO (Gene Ontology) (http://geneontology.org/) terms enrichment analysis (17). Pathway analysis involved by DEGs was performed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.kegg.jp/) database using the KOBAS web server (http://kobas.cbi.pku.edu.cn/) (18). \( P < 0.05 \) was selected as the cut-off.
Associations between the enriched GO and KEGG pathway terms. Cytoscape software 3.5.1 (http://www.cytoscape.org/) was used to investigate the associations between significantly enriched GO and KEGG pathway terms (19). To visualize the results using Enrichmentmap Plug-in of Cytoscape (20), the criteria were node \( P \) value cut-off = 0.001, false discovery rate \( q \) value cut-off = 0.1, edge cut-off (similarity) = 0.5. The associations between two terms were calculated according to the overlapped DEGs, and terms shown strong associations were used to construct the association networks.

FIGURE 1—Volcano boxplot of DEGs between two sets of samples (A) and hierarchical clustering heatmap of the top 50 DEGs (B). (A) The red points represent upregulated genes screened on the basis of \(|\text{LogFC}| > 0.75 \) and \( P \) value < 0.05. The green points represent downregulation of the expression of genes screened on the basis of \(|\text{LogFC}| < 0.75 \) and \( P \) value < 0.05. The black points represent genes with no significant difference; (B) Red indicates that gene expression is relatively upregulated, green indicates that gene expression is relatively downregulated, and black indicates no significant changes in gene expression.
Interactions between differential expressed genes. The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, http://string-db.org/) online database was used to identify the PPI between proteins encoded by DEGs (21). Cytoscape software was used to construct the PPI network (19). The network were shown with nodes and edges. The top 10 nodes were screened out by degree filter and then visualized using plug-in Cytohubba (22) of Cytoscape. DEGs with high degree will be considered as hub genes that might play crucial roles in exercise mediated regulatory processes, as degree means the number of nodes linked to a certain node.

Module analysis of interaction network. Proteins in the same module of a PPI network usually have the same biological processes and functions by co-expression. In this study, we used plug-in MCODE of Cytoscape to mine the modules from the PPI network with score >10 (23). Thereafter, plug-in Bingo was used to annotate the functional group of those genes in the modules based on the hypergeometric distribution (corr $P < 0.01$, DEG count $\geq 2$) (24), and KOBAS web server was used to identify the KEGG pathways.

RESULTS

Screened differential expressed genes. When the data set GSE62628 from Pedersen et al.’s previous report (14) was screened using the limma package, 315 DEGs were obtained. Among them, 294 upregulated genes and 21 downregulated genes were identified. The differential expression of multiple genes from the two sets of sample data is shown in Figure 1A. The cluster heatmaps of the top 50 DEGs are shown in Figure 1B.

Function of differential expressed genes. GO functional enrichment of all DEGs with $P < 0.05$ and DEG count $\geq 2$ were obtained using the DAVID online tool. Results of GO analysis were divided into three functional groups, i.e., molecular function (MF), cellular component (CC) and biological process (BP) (see Table, Supplemental Digital Content 1, Details of significant GO and KEGG pathway terms of DEGs, http://links.lww.com/MSS/B924). The top 15 enrichments in each functional group are shown in Figure 2. In the BP group, genes were mainly enriched in immune and inflammatory responses.
response, and positive regulation of ERK1 and ERK2 cascade. In the CC group, genes were mainly enriched in extracellular space, extracellular region, and the external side of the plasma membrane. In the MF group, genes were mainly enriched in chemokine activity, cytokine activity, and binding function. As for the upregulated DEGs, they were significantly enriched in 292 terms, including 209 BP terms, 41 CC terms, and 42 MF terms. DEG downregulated were significantly enriched in 9 terms, including 5 BP terms, 1 CC term (mitochondrion) and 3 MF terms (oxidoreductase, NAD, and oxidoreductase activity). Under the same criteria, DEGs upregulated were significantly enriched in 45 KEGG pathway terms, and downregulated DEGs were significantly enriched in 4 KEGG pathway terms (histidine, beta-alanine, arginine, and proline metabolism and metabolic pathways) (Fig. 3).

**Associations between enriched terms.** Among the 337 terms enriched by upregulated DEGs, 103 GO terms and 27 KEGG pathway terms were strongly associated with each other, and these terms were mainly related with the membrane or extracellular region. Among the 13 terms enriched by downregulated DEGs, no GO terms or KEGG pathway terms were strongly associated with each other (see Figure, Supplemental Digital Content 2, Functional association networks of DEGs, http://links.lww.com/MSS/B925).

**Protein–protein interaction network.** The PPI network was constructed using the STRING database (the minimum

FIGURE 3—Significant pathway enrichment of upregulated (A) and downregulated (B) DEGs. Blue represents the signaling pathway, red represents upregulated genes, and green represents downregulated genes.
required interaction score was set at the highest confidence of 0.900) and Cytoscape software, with a total of 315 DEGs, including 294 upregulated and 21 downregulated genes. After removing the disconnected nodes in the network, a complex network of DEGs was constructed, as shown in Figure 4. The 17 most significant genes showing significant interaction were HBB, ZWINT, WNT2B, SPP1, HBA2, NUF2, ALDH1A1, FZD10, MMP7, MUC16, MUC1, OMD, OGN, AOX1, ADH1B, HBG2, and TTK. The top 10 nodes were C3, Kng1, C3ar1, Ptafr, Fgg, Alb, Pf4, Orm1, Aldh3b1, and Apob as shown in Figure 5.

**Module analysis of interaction network.** MCODE was used to mine the densely connected modules in protein–protein interaction network. With a score >10, one module was screened out (Fig. 6). This module contains 41 nodes, and all hub genes were included. By using BINGO to perform functional annotation, 192 terms, including 155 BP terms, 7 CC...
terms, and 30 MF terms, were screened out with the criteria corr $P < 0.01$ and DEG count $\geq 2$. Five KEGG pathways were enriched using the DAVID online tool with the criteria $P < 0.01$ and DEG count $\geq 2$ (see Table, Supplemental Digital Content 3, Top 5 significant enrichments for each functional group in module function, http://links.lww.com/MSS/B926). Biological functional enrichment analysis showed that genes in this module were significantly enriched in response to stimuli, response to wounding, immune system process, and inflammatory and immune response. Complement and coagulation cascades, Staphylococcus aureus infection, cytokine–cytokine receptor interaction, chemokine signaling pathway and phagosomes were mainly involved. Moreover, C3, C3ar1, Kng1, Ptafr, and Fgg may be the critical genes in the complement and coagulation cascades pathway and S. aureus infection pathway. These results indicate that voluntary exercise may exert positive effects on melanoma tissues by regulating inflammation and the immune response. Because the mechanistic effects of exercise in relation to clinical treatment and tumor-specific outcomes are largely unknown, further experimental research targeting these genes and signaling pathways are warranted to enhance the etiology and treatment of melanoma, and to reveal the potential of exercise in the perioperative period. Moreover, these investigations may also lay the theoretic foundation for the application of these targets in clinical translation medicine.

Recently preclinical exercise-oncology studies have documented similar exercise-related protection against tumor incidence and growth (3,27–29); however, only a few studies have investigated the anti-inflammatory and immunomodulation effects within tumor tissues induced by exercise. For example, after implantation with four T1 cells, female BALB/c mice received 6 wk aerobic interval training on a treadmill (5 d·wk$^{-1}$) consisting...
of ten 2-min intervals of running at 70% VO₂max separated by 2 min of active recovery at 50% VO₂max. The results showed that exercise significantly decreased tumor volume, and increased the intratumoral oncostatin M and TNF-α levels (30). In MC4L2 tumor-bearing mouse model, 6 or 14 wk of progressive treadmill running (5 d·wk⁻¹) led to a significant reduction in the level of IL-6, tumor volume, and growth, which highlighted the critical role of postimplantation exercise (31). Zhang et al. also found that 42-d moderate swimming (8 min·d⁻¹, 5 d·wk⁻¹) posttransplantation significantly reduced the relative tumor weight and lung metastases while increasing the mean median survival time by 9 d in Hepa1–6 tumor-bearing mice, and the proportion of CD4+ CD25+ Foxp3+ Treg cells in tumor tissues were significantly decreased in exercised mice (32). Similar results can also be found in Ehrlich, in EL-4, and in HCC-LM3 tumor-bearing mice models (33–35). Therefore, our findings fit well with these previous studies and the hypothesis that exercise ameliorates the immune and inflammatory responses in tumors.

Current evidence from preclinical studies indicates that moderate to high-intensity aerobic exercise is superior to light exercise, when aiming to target intrinsic tumor factors (36). However, limited consensus can be drawn from animal studies regarding recommendations for the optimal mode of exercise, as well as volume, duration, and intensity in humans. As mentioned above, nearly all studies utilized a protocol with moderate intensity and 5 d·wk⁻¹ frequency for tumor-bearing animals, with mixed results. Moreover, 80% of maximal workload swimming for 1 h·d⁻¹, 5 d·wk⁻¹, over 6 wk did not reduce the weight and volume of tumors in Ehrlich tumor-bearing mice, whereas 50% maximal workload reduced tumor volume (33). A recent study investigating the effects of 9 wk of moderate (8 min·d⁻¹) and overload (16 and 32 min·d⁻¹) swimming on Hepa1–6 tumor initiation and progression reported similar results (35). Therefore, it is possible that voluminous high intensity exercise may compromise the inflammatory and immunomodulation effects of exercise that benefit cancer outcomes. As for the present study, voluntary wheel running was adopted in the original experiment, intensity was not controlled, and only the average running distance was reported (14), thus, it is difficult to draw a definite conclusion regarding the exercise intensity, dose, and duration. However, taking the aforementioned clues and the susceptibility of tumor-bearing animals into consideration, a programmed aerobic exercise protocol with moderate intensity and volumes, and higher exercise frequency (5 d·wk⁻¹), seems effective at reducing tumor growth in animals.

Among the critical genes screened in the current study, C3, C3ar1, Kng1, and Fgg were enriched in complement and coagulation cascades, which have been identified in multiple cancers, suggesting an essential role of this pathway in cancer biology (37). Complement protein C3 had previously been suggested as a prognostic factor of lung cancer (38). Jandl et al. (39) found that radioimmunotherapy leads to increased C3 deposition in melanoma tumor, and activates the host immune system, leading to a suppression of the tumor. The effects of C3ar1 expression on melanoma is not clear, but Nabizadeh’s group (40) showed that development and growth of B16-F0 melanomas is retarded in mice lacking C3aR (complement C3a receptor), while growth of established melanomas can be arrested by the C3aR antagonist. There are no reports about the relationship between melanoma and Kng1 to date. A previous study hypothesized that the high molecular weight kinogen may be considered as the angiogenic contributor in melanoma progression (41), thus the inhibition of it may help to decrease the tumor burden. Fgg encodes the gamma subunit of the fibrinogen. Animal studies demonstrate that different components of the coagulation system such as fibrinogen can promote tumor progression (42). As for the effects of exercise, ours is the first study we are aware of that suggests exercise modulates these genes within tumor tissues. Finally, it has been established that oxidized Ptafr agonists augment murine B16F10 melanoma tumor growth in a platelet-activating factor receptor-dependent manner because of its effects on host immunity (43), but the relationship between exercise and Ptafr is still not clear.

Exercise-induced alterations in the systemic milieu influence key regulatory mechanisms in the tumor microenvironment, such as metabolism and immune regulation, and thus was considered to have a cumulative antitumorigenic effect (44). Although the majority of current studies support this tumor-suppressing effect of exercise, we admit that a considerable publication bias may exist favoring positive findings. And, little consensus can currently be drawn from these studies regarding recommendations for the optimal volume, intensity, and duration of aerobic exercise. To further study the mechanism of exercise-dependent control of melanoma tumor tissue, we systematically re-analyzed the gene expression profile. However, there are still two major limitations in the present study that could be addressed in future research. First, the sample size for gene profile analysis was small which might lead to a certain rate of false positive results despite our attempts to control for this with standardized analyzing processes and strict screening thresholds. Second, it is difficult to draw a clear conclusion concerning the detailed effects of the intervention because of the design of the original experiment. Only running distance was reported in the melanoma experiment, thus while we can discuss exercise in general, we cannot further distinguish how exercise volume, intensity, and duration may affect these candidate genes and pathways in tumor tissues.

In our humble opinion, further studies are warranted to investigate the associations between exercise and melanoma after detailed adjustment for sun exposure and ultraviolet radiation-related skin damage. The biological mechanisms by which exercise affects melanoma cancer processes require further investigation, since the associations between exercise and melanoma (particularly the intratumoral alterations) are critical to propose recommendations, and the current mechanisms through which this intervention operates (including the mode of exercise, as well as the volume, intensity, and duration) are less clearly elucidated. Moreover, more efforts are
needed to explore the impact of exercise throughout the course of life on melanoma risk.

CONCLUSIONS

The objective of the present study was to improve our understanding of the molecular mechanisms underlying the regulatory effects of exercise on melanoma tissue through bioinformatics analysis. We identified 294 upregulated genes and 21 downregulated genes in the exercise group and 10 candidate hub genes including C3, Kng1, C3ar1, Ptafr, Fgg, Alb, P4f, Orm1, Aldh3b1, and Apob for exercise were predicted. C3, C3ar1, Kng1, Ptafr, Fgg may be the critical genes in the complement and coagulation cascade pathways and S. aureus infection pathway. These findings indicate that exercise ameliorated the immune response and inflammatory response in melanoma tissue.

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