Research Article

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Identification of immune-related genes in thymus of breast cancer mouse model exposed to different calorie restriction

Farklı Kalori Kısıtlaması Uygulanmış Meme Kanseri Model Farenin Timusundaki İmmün Sistem İlişkili Genlerin Belirlenmesi

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Abstract

Introduction: In the present study, RNA sequencing-mediated transcriptome analysis was performed in order to elucidate the molecular mechanisms of the immune response for different types of calorie restriction (CR) application using MMTV-TGF-α breast cancer mouse model.
Methods: Animals were applied to three different dietary regiments; ad libitum (AL), chronic calorie restriction (CCR) and intermittent calorie restriction (ICR). Using thymus tissues, 6091 differentially expressed genes (DEGs) were identified in three dietary groups. After clustering of total of 6091 DEGs using Gene Ontology (GO) categories, a total of 400 genes were identified to be involved in immune system process (GO:0002376) GO categories. KEGG pathway and gene co-expression network analysis of these immune-related DEGs were done using String database. The results were confirmed with measuring mRNA expression levels of four selected immune-related DEGs genes (Casp3, Thy1, IL-16 and CD4) using quantitative real-time PCR (qPCR).
Results: The expression levels of immune-related genes were different in three RNA-seq data.
Conclusion: The results provide useful information to investigate the immune-related transcriptional profiling in thymus tissue of breast cancer mouse model applied to two different types of CR and to identify the specific functional immune related genes in response to CR during cancer development.

Keywords: Intermittent calorie restriction; Chronic calorie restriction; Thymus; RNA-seq; Immune-related genes.

Öz

Amaç: Bu çalışmada, MMTV-TGF-a meme kanseri fare modeli kullanılarak, farklı kalori kısıtlaması (KK) uygulamaları için immün yanıtın moleküler mekanizmalarını açıklamak amacıyla RNA sekanslama aracılıklı transkriptom analizi yapılmıştır.
Gereç ve Yöntem: Farelere üç farklı beslenme uygulanmıştır; ad libitum (AL), kronik kalorili kısıtlama (KKK) ve aralıklı kalori kısıtlaması (AKK). Timus dokuları alınarak üç beslenmedeki diferansiyel eksprese olan genlerin toplamda 6091 tane olduğu tanımlanmıştır. Bu genler genler Gen Ontolojisi (GO) kategorileri kullanılarak sınıflandırılmıştır. İmmün sistem proses (GO: 0002376) GO kategorisinde toplam 400 gen tespit edilmiştir. Bu genler için KEGG yolak ve ortak ifade ağ analizi, String veritabanı kullanılarak yapılmıştır. Daha sonra, kantitatif gerçek zamanlı PCR kullanılarak seçilmiş dört immün sistemde görevli geninin (Casp3, Thy1, IL-16 ve CD4) mRNA ekspresyon seviyeleri ölçülmüştür.

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Sonuç: Üç RNA-seq datasında immün sistemle ilişkili genlerin ekspresyon seviyelerinin farklı olduğu gözlemlenmiştir.
Tartışma: Sonuçlar, iki farklı kalori kısıtlaması uygulanan meme kanseri fare modelinin timus dokusunda immün sistem ile ilgili transkripsiyonel profili araştırmak için ve kanser gelişimi sırasında kalori kısıtlamasına yanıt olarak spesifik fonksiyonel immün sistem ilişkili genleri tanımlamamıza yardımcı olacak bilgiler sağlamaktadır.

Anahtar kelimeler: Aralıklı kalori kısıtlaması; Kronik kalori kısıtlaması; Timus; RNA sekanslama; İmmün sistem ilişkili genler

Introduction

Calorie restriction (CR) which involves a sustained, moderate reduction in calorie intake compared to ad libitum (AL) feeding is a dietary regimen [1]. CR has proven to be an effective method in multiple species to decrease the incidences of chronic disease [2, 3], hypertension, heart disease, kidney disease, and neurological dysfunction and increases sensitivity to insulin and the lifespan [4–6]. Two common CR types are chronic calorie restriction (CCR) and intermittent calorie restriction (ICR) which involves strong restriction on 1 or 2 days per week [7, 8].

MMTV-TGF-α transgenic mice have particular value in age-related mammary tumor (MT) development studies. These mice have been reported to develop MT in the second year of their life [9] due to over expression of TGF-α, epidermal growth factor, which also plays a critical role in development of human breast cancer [10]. In several animal studies, the protective effect of CR on MT development has been shown [11–13]. However, the results of different CR types with respect to cancer prevention is controversial. Some studies suggested CCR to be more preventive for MT development compared to ICR [14, 15], while other studies showed ICR is more effective than CCR [7, 16, 17].

The thymus is a primary lymphoid organ and a place of T-cell differentiation and maturation [18]. Importantly, the changes in the adaptive immune system increase with CR and aging [19, 20]. In rodents and in non-human primates, CR increases production of native T cells [21] and T cell proliferation [22], but these functions also decrease with aging. Studies have reported that although inflammatory cytokines such as IL-6, TNF-α and IFN-g were decreased by CR [22, 23], they were not changed with aging [24]. In addition, age-associated dysfunction of natural killer cells has been reported in mice [25] and human studies [26]. On the other hand, although there are many studies have been conducted to understand the roles of immune cells in cancer development and their responses to CR, the exact molecular mechanism of this process has not been clearly understood yet. Especially, the roles of specific gene(s) involved in immune system responses to CR needs be clarified. Therefore, the aim of the present study was by using RNA-seq transcriptome analysis method to identify the immune related genes in thymus of breast cancer mouse model in response to different types of CR application.

Materials and methods

Mouse model and tissue accession

In this study, MMTV-TGF-α (C57/BL6) female mice overexpress human TGF-α, a part of epidermal growth factor receptor (EGFR)/ErbB cascade which is known to play a role in the development of human breast cancers, were used [10]. Mice colony were maintain using a breeding protocol and genotyping assay at Yeditepe University Animal Facility as previously described [12]. At 10 weeks of age female MMTV-TGF-α mice were assigned to one of three fed groups: AL, chronic caloric restricted (CCR) and intermittent caloric restricted (ICR). The CCR group received 85% of the daily food consumption of AL mice, in other words they were applied to 15% caloric restriction. The ICR group was fed AL for 3 weeks then following week 60% caloric restriction compared to AL were applied. Mice diets (Altromin TPF1414) were purchased from Kobay AS (Ankara, Turkey). All mice had free access to water. Food intakes were determined daily and body weights weekly. Animals were also checked by expert veterinarian on a regular basis at least once a week. Mice were euthanized after overnight fasting at the age of 17 or 18 weeks old. Mice in ICR group were euthanized after three weeks of AL feeding.

RNA isolation, library preparation and sequencing

Total RNA was isolated from the thymus tissue samples of three different individual mice in each different dietary groups. Preparation of cDNA library and RNA sequencing were performed by Beijing Institute of Genomics [27]. RNA-seq data has been deposited in NCBI Gene Expression Omnibus database under accession number GSE95371.

Statistical analysis and functional annotation of gene expression

The sequencing reads were aligned to GRCm38 mouse genome (Ensemble database) using the splice-aware

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TopHat aligner [28]. Filtered mapped reads were analyzed using the Cufflinks package with default parameters and the following additions. Differentially expressed genes (DEGs) were determined based on false discovery rate (FDR)-adjusted p-value <0.05 as calculated by Cuffdiff [29]. All of the data produced by a Cuffdiff analysis was visualized and integrated with R [30].

The DEGs have mapped to GO terms in the database (http://www.geneontology.org/, Panther GO-Slim), calculated gene numbers for immune system process (GO:0002376), using a hypergeometric distribution compared with the genome background. KEGG analysis and gene co-expression network was visualized by the String database (www.strindb.org) [31].

Table 1: The information of the primer pairs used to analyze gene expression levels by qPCR.

Gene names	Primer sequences
Thy-1	5′TCTCAGGCACCCTTGGGATA 3′
	5′GTAGTCGCCCTCATCCTTGG 3′
IL-16	5′ATGGTGCTCCCAGAGTTTAC 3′
	5′CTGAATGGCTGAGGCTACTT 3′
CD4	5′TGGATCAAAGGGCAGTGTATAG 3′
	5′GCAGCCTCTCAGTCTTCA TT 3′
Casp3	5′AGCTTGGAACGGTACGCT 3′
	5′AGA TCCCAGAGTCCACTGAC 3′
Gapdh	5′ACT CCA CTC ACG GCA AAT TC3′
	5′CAGTAGACTCCACGACATACT C3′

GAPDH was used as an internal control.

Validation of RNA-seq genes by qPCR

Four unigenes with significant expression difference (*Casp3*, *Thy1*, *IL-16* and *CD4*) involved in immune system were chosen for validation by quantitative real-time PCR (qPCR). Using GM SYBR Green qPCR Kit qPCR was performed with the LightCycler Nano Real-Time System (Roche, Switzerland). The thermal cycling conditions were 95°C for 2 min, 45 cycles at 95°C for 20 s for denaturation, and 58°C and 72°C for 30 s and 45 s for annealing and extension. The $2^{-\Delta CT}$ method was used to calculate relative gene expression levels in each sample. Primer sequences were designed using IDT and Primerblast programs and Gapdh was used as an internal control (Table 1).

Results

Summary of RNA-seq analysis

In our previous project, three sequencing libraries were constructed from thymus tissues of MMTV-TGF-α mice in AL, CCR and ICR diet groups for RNA-seq. Approximately 127 million clean reads were mapped to GRCm38 mouse assembly in the Ensemble database with TopHat. Ratio of mapped reads approximately 92.5%. Corrected FPKM values were subjected to analysis of DEGs, a total of 6091 significantly DEGs were identified in three different diet groups, by using corrected p-value <0.05 as the filter [27]. The 2821, 2825 and 445 significantly DEGs were detected between diet groups AL-CCR, CCR-ICR, AL-ICR, respectively ($p < 0.05$). According to these results; 916, 1877 and 200 genes were up-regulated and 1905, 948 and 245 genes down-regulated in DEGs between the diet groups AL and CCR, CCR and ICR, AL and ICR, respectively [27].

Identification of immune-related genes

DEGs obtained from these libraries, between AL and CCR, AL and ICR, CCR and ICR diet groups ($p < 0.05$), were classified according to cellular components, biological processes and molecular functions GO main categories. The biological process category includes "immune system process (GO:0002376)" GO category. The DEGs obtained from these libraries were further subjected to GO functional enrichment analysis, which provided to describe immune-related genes in "immune system process (GO:0002376)" term. In GO:0002376 term, 188 of 2821, 36 of 445, 176 of 2825 genes differential expressed between AL-CCR, AL-ICR, CCR-ICR diet groups, respectively.

Besides, 67, 92 and 17 of immune-related genes were up-regulated and 121, 19 and 84 genes down-regulated between AL and CCR, CCR and ICR, AL and ICR fed groups, respectively. These immune-related genes were shown according to the log2Fold change <–2 and log2Fold change >2 value (Tables 2–4).

Table 2: Immune-related genes between AL and ICR filtered log2Fold change <–2 and log2Fold change >2 value.

Gene names	Sample_1	Sample_2	log2(Fold_change)	p-Value
Pcolce	AL	ICR	3.19965	5E-05
Spint1	AL	ICR	–2.13611	0.0123
Col15a1	AL	ICR	–2.54181	0.00275

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Table 3: Immune-related genes between AL and CCR filtered log2Fold change <−2 and log2Fold change >2 value.

Gene names	Sample_1	Sample_2	log2(Fold_change)	p-Value
Tns1	AL	CCR	2.81267	5.00000E−05
Cfh	AL	CCR	3.86202	5.00000E−05
Csrp2	AL	CCR	2.43296	0.0015
Msrb3	AL	CCR	3.25099	0.0004
Gpx3	AL	CCR	3.71675	5.00000E−05
Cd79b	AL	CCR	2.05737	0.00135
Ighv3-6	AL	CCR	3.75866	0.00065
Ighv1-19	AL	CCR	2.52651	0.0126
Tns2	AL	CCR	3.02069	5.00000E−05
Adipoq	AL	CCR	3.42312	5.00000E−05
Adgrf5	AL	CCR	3.63443	5.00000E−05
C4b	AL	CCR	3.22915	5.00000E−05
Slc27a2	AL	CCR	4.29046	0.00045
Acss2	AL	CCR	2.54708	5.00000E−05
Cd302	AL	CCR	2.63002	0.0167
S100a6	AL	CCR	2.57191	5.00000E−05
Adgrl4	AL	CCR	2.50311	0.0095
S100a1	AL	CCR	3.33725	5.00000E−05
Hspb1	AL	CCR	3.02974	0.00195
Cd36	AL	CCR	4.31277	5.00000E−05
Sh2b2	AL	CCR	3.59971	0.00095
March8	AL	CCR	2.13474	0.00235
Igkv4-72	AL	CCR	2.41746	0.00505
Cebpa	AL	CCR	2.84059	5.00000E−05
Acsm3	AL	CCR	5.10879	5.00000E−05
Fcgrt	AL	CCR	2.35414	5.00000E−05
Slc27a1	AL	CCR	3.44268	5.00000E−05
Fhl1	AL	CCR	4.49713	5.00000E−05
Cybb	AL	CCR	2.19663	0.00135
Iglc1	AL	CCR	2.85077	5E-05
Pcolce	AL	CCR	3.47498	5E-05
Zap70	AL	CCR	−3.11197	5.00000E−05
Slamf6	AL	CCR	−2.81828	0.00025
Mr1	AL	CCR	−2.07319	5.00000E−05
Lcp2	AL	CCR	−2.24039	0.0001
Grap	AL	CCR	−2.14867	4
Skap1	AL	CCR	−3.86327	5.00000E−05
Itk	AL	CCR	−3.41276	5.00000E−05
Nlk	AL	CCR	−2.05006	0.00035
Stat5b	AL	CCR	−2.19968	5.00000E−05
Spata13	AL	CCR	−2.13382	0.00025
Elf1	AL	CCR	−2.07977	5.00000E−05
Grap2	AL	CCR	−2.91878	5.00000E−05
Sla	AL	CCR	−3.65399	5.00000E−05
Def6	AL	CCR	−3.12154	5.00000E−05
Crip3	AL	CCR	−2.53914	0.0001
Vav1	AL	CCR	−2.84485	5.00000E−05
Tagap1	AL	CCR	−2.19823	0.00025
Ppp1r14b	AL	CCR	−2.14199	5.00000E−05
Dntt	AL	CCR	−5.67572	5.00000E−05
Cd5	AL	CCR	−3.64272	5.00000E−05
Lef1	AL	CCR	−3.91587	5.00000E−05
Sit1	AL	CCR	−4.22883	0.00145
Lck	AL	CCR	−3.86171	5.00000E−05
Txk	AL	CCR	−2.65584	5.00000E−05
Gimap9	AL	CCR	−2.36907	5.00000E−05
Cd8a	AL	CCR	−4.8846	5.00000E−05

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Table 3 (continued)

Gene names	Sample_1	Sample_2	log2(Fold_change)	p-Value
Cd4	AL	CCR	−3.77405	5.00000E−05
Il21r	AL	CCR	−2.81807	5.00000E−05
Spint2	AL	CCR	−2.05501	5.00000E−05
Ccl25	AL	CCR	−3.01229	5.00000E−05
Casp3	AL	CCR	−3.05056	5.00000E−05
Rltpr	AL	CCR	−4.35955	5.00000E−05
Thy1	AL	CCR	−4.29803	5.00000E−05
Trbc1	AL	CCR	−4.64904	5E−05
Trac	AL	CCR	−2.47246	5E−05
Trbc2	AL	CCR	−4.63055	5E−05
Colq	AL	CCR	−2.3138	5E−05

Table 4: Immune-related genes between CCR and ICR filtered log2Fold change <− 2 and log2Fold change >2 value.

Gene names	Sample_1	Sample_2	log2(Fold_change)	p-Value
Cd2	CCR	ICR	2.01665	5.00000E−05
Plekhg2	CCR	ICR	2.02515	5.00000E−05
Elf1	CCR	ICR	2.04993	5.00000E−05
Casp8	CCR	ICR	2.05028	5.00000E−05
Tagap1	CCR	ICR	2.20259	0.0001
Stat5b	CCR	ICR	2.22228	5.00000E−05
Spata13	CCR	ICR	2.24812	0.00015
Slfn2	CCR	ICR	2.32184	5.00000E−05
Slamf6	CCR	ICR	2.33621	3
Cd84	CCR	ICR	2.3897	5.00000E−05
Colq	CCR	ICR	2.40085	0.0001
Mr1	CCR	ICR	2.41042	5.00000E−05
Lcp2	CCR	ICR	2.44881	5.00000E−05
Clec2i	CCR	ICR	2.4591	5.00000E−05
Casp3	CCR	ICR	2.51841	5.00000E−05
Grap	CCR	ICR	2.52795	0.00055
Gimap9	CCR	ICR	2.57845	5.00000E−05
Crip3	CCR	ICR	2.82336	5.00000E−05
Cd69	CCR	ICR	2.85433	0.0003
Trac	CCR	ICR	2.93217	5E−05
Grap2	CCR	ICR	2.95583	5.00000E−05
Zap70	CCR	ICR	2.99931	5.00000E−05
Ccl25	CCR	ICR	3.04315	5.00000E−05
Txk	CCR	ICR	3.05417	5.00000E−05
Il21r	CCR	ICR	3.14165	5.00000E−05
Vav1	CCR	ICR	3.16942	5.00000E−05
Cd5	CCR	ICR	3.22315	5.00000E−05
Itk	CCR	ICR	3.43302	5.00000E−05
Skap1	CCR	ICR	3.52315	5.00000E−05
Lef1	CCR	ICR	3.56139	5.00000E−05
Def6	CCR	ICR	3.7327	5.00000E−05
Lck	CCR	ICR	3.82005	5.00000E−05
Sla	CCR	ICR	3.95902	5.00000E−05
Cd4	CCR	ICR	4.03836	5.00000E−05
Sit1	CCR	ICR	4.06077	0.0013
Rltpr	CCR	ICR	4.09504	5.00000E−05
Thy1	CCR	ICR	4.18136	5.00000E−05
Trbc1	CCR	ICR	4.83734	5E−05
Trbc2	CCR	ICR	4.84636	5E−05
Cd8a	CCR	ICR	5.06282	5.00000E−05
Slamf1	CCR	ICR	5.21796	0.0147

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Table 4 (continued)

Gene names	Sample_1	Sample_2	log2(Fold_change)	p-Value
Dntt	CCR	ICR	5.82443	5.00000E–05
Ighv3-6	CCR	ICR	–4.416	0.00345
Pla2g7	CCR	ICR	–2.06739	0.0029
Abhd4	CCR	ICR	–2.07835	0.0002
Ighv1-82	CCR	ICR	–2.1234	0.0163
Fcgrt	CCR	ICR	–2.12974	5.00000E–05
C2	CCR	ICR	–2.1523	5E–05
Col4a1	CCR	ICR	–2.1836	5.00000E–05
Msrb3	CCR	ICR	–2.31109	0.0009
S100a6	CCR	ICR	–2.3115	0.0002
S100a1	CCR	ICR	–2.31869	5.00000E–05
Ighv1-26	CCR	ICR	–2.31928	0.0054
Slc27a2	CCR	ICR	–2.41265	0.00045
Igkv1-110	CCR	ICR	–2.52224	0.00035
Cd302	CCR	ICR	–2.54036	0.0151
Adgrf5	CCR	ICR	–2.55154	5.00000E–05
Slc27a1	CCR	ICR	–2.56109	5.00000E–05
Fbln1	CCR	ICR	–2.57612	0.0033
Crip2	CCR	ICR	–2.59587	5.00000E–05
Cebpa	CCR	ICR	–2.61195	5.00000E–05
C4b	CCR	ICR	–2.74532	0.00055
Acsm3	CCR	ICR	–2.74966	0.00245
Cd55	CCR	ICR	–2.76376	0.00015
Sh2b2	CCR	ICR	–2.90785	0.0018
Tns1	CCR	ICR	–3.05026	5.00000E–05
Fhl1	CCR	ICR	–3.12463	5.00000E–05
Adipoq	CCR	ICR	–3.1317	5.00000E–05
Cd36	CCR	ICR	–3.14103	5.00000E–05
Gpx3	CCR	ICR	–3.31306	5.00000E–05
Cfh	CCR	ICR	–3.4394	5.00000E–05
Csrp3	CCR	ICR	–3.63321	0.00545
Cxcl13	CCR	ICR	–3.66985	0.00405
Adgrl4	CCR	ICR	–3.68165	0.0018
Tns2	CCR	ICR	–3.77329	0.0001
Iglv1	CCR	ICR	–3.8441	0.0001
Cryab	CCR	ICR	–3.97295	0.0014
Hspb7	CCR	ICR	–4.50642	0.00925

Pathway and gene network analysis

A KEGG pathway analysis was performed in identified immune-related DEGs pertaining to the AL-CCR, AL-ICR, CCR-ICR diet groups. When filtered by FDR, the most significant two KEGG terms were antigen processing and presentation (pathway ID: 04612), T cell receptor signaling pathway (pathway ID: 04660) for between AL-CCR DEGs; T cell receptor signaling pathway (pathway ID: 04660) and TNF signaling pathway (pathway ID: 04668) for between CCR-ICR DEGs; tuberculosis (pathway ID: 05152) and toxoplazmosis (pathway ID: 05145) for between AL-ICR DEGs. Some immune-related KEGG pathways and the DEGs count were shown in Table 5.

To identify co-expression profiles of AL-CCR, AL-ICR, CCR-ICR immune-related DEGs, a gene co-expression network was generated using gene expression data from DEGs identified in this study (Figure 1A–C).

Nodes indicated the immune-related DEGs and edges presented the interaction between immune-related DEGs (Table 6).

Validation with qPCR

To validate the reliability of the expression of immune-related DEGs obtained from RNA-seq analysis, qPCR was used to dissect dynamic change in gene expression level in four genes; *Casp3, Thy1*, *IL-16* and *CD4*. The results of RNA-seq and qPCR for four DEGs were shown in Figures 2 and 3, respectively. According to the results of RNA-seq and qPCR; Casp3, *Thy1*, *IL-16* and *CD4* were up-regulated

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Table 5: The results of KEGG pathway analysis of AL-CCR, CCR-ICR and AL-ICR DEGs.

KEGG pathways	AL-CCR		CCR-ICR		AL-ICR	
	Gene count	FDR	Gene count	FDR	Gene count	FDR
T cell receptor signaling pathway	12	1.73E-09	11	3.17E-08	–	–
TNF signaling pathway	9	1.53E-06	10	1.71E-07	–	–
Natural killer cell mediated cytotoxicity	10	2.54E-07	9	2.25E-06	–	–
Antigen processing and presentation	12	5.1E-11	5	0.00106	3	0.00442
B cell receptor signaling pathway	8	9.82E-07	6	0.000115	–	–

in AL fed group compared with ICR and CCR diet groups (Figures 2 and 3).

Discussion

Thymus is a primary lymphoid organ in the immune system that is largely replaced with fat at an early age independent of adiposity or disease. Given that the function of thymus is to establish and maintain T cell arm of immunity and not to regulate energy homeostasis, the adipocytes develop within the thymic [5, 18]. Thymus and other lymphoid organs react to nutrition deficiency more rapidly than most of the other organs [32, 33]. Several studies from animal models show that CR has a significant impact on the immune system. Most of the studies suggest that CR improves many parameters of immune responses [20, 34, 11].

In this study, RNA were isolated from thymus tissue of AL, chronically calorie restriction (CCR) and ICR fed MMTV-TGF-α mice from 10 weeks of age to 17 weeks of age or 18 weeks of age. RNA-seq resulted in 6091 significantly DEGs were obtained between three fed groups;

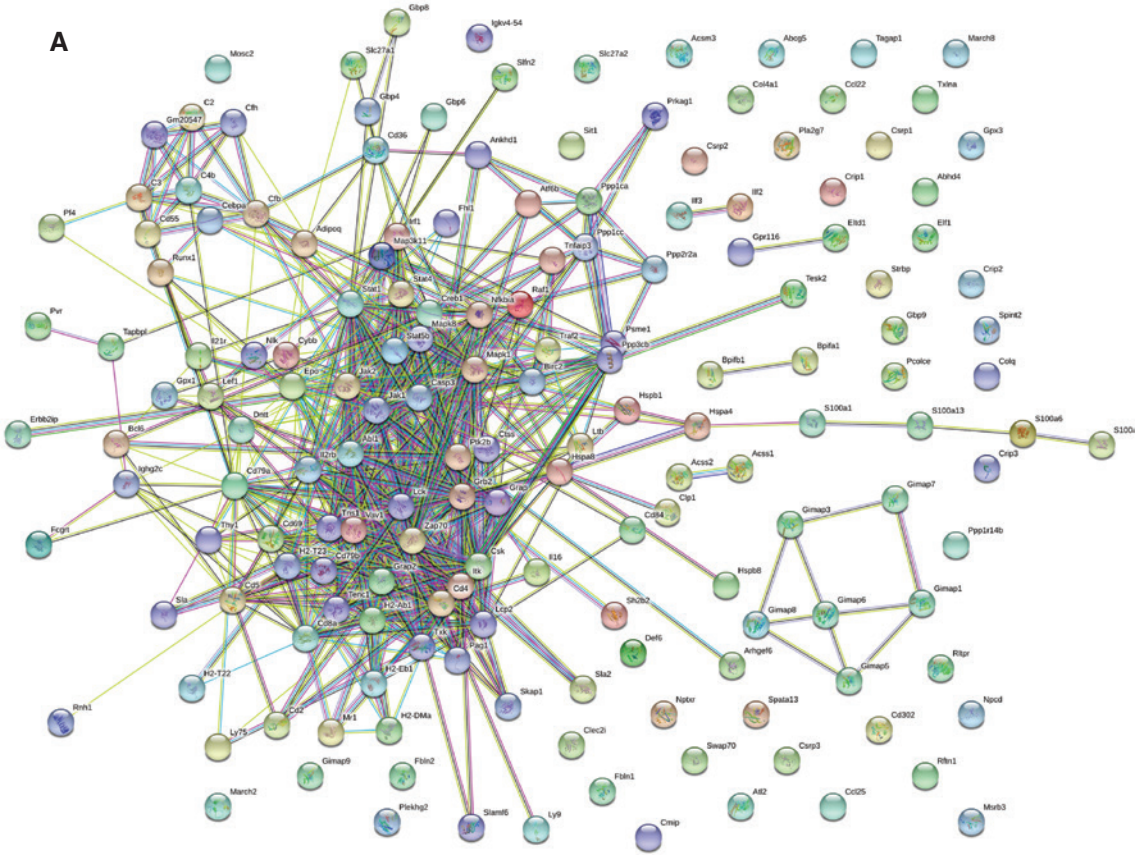


Figure 1: (continued)

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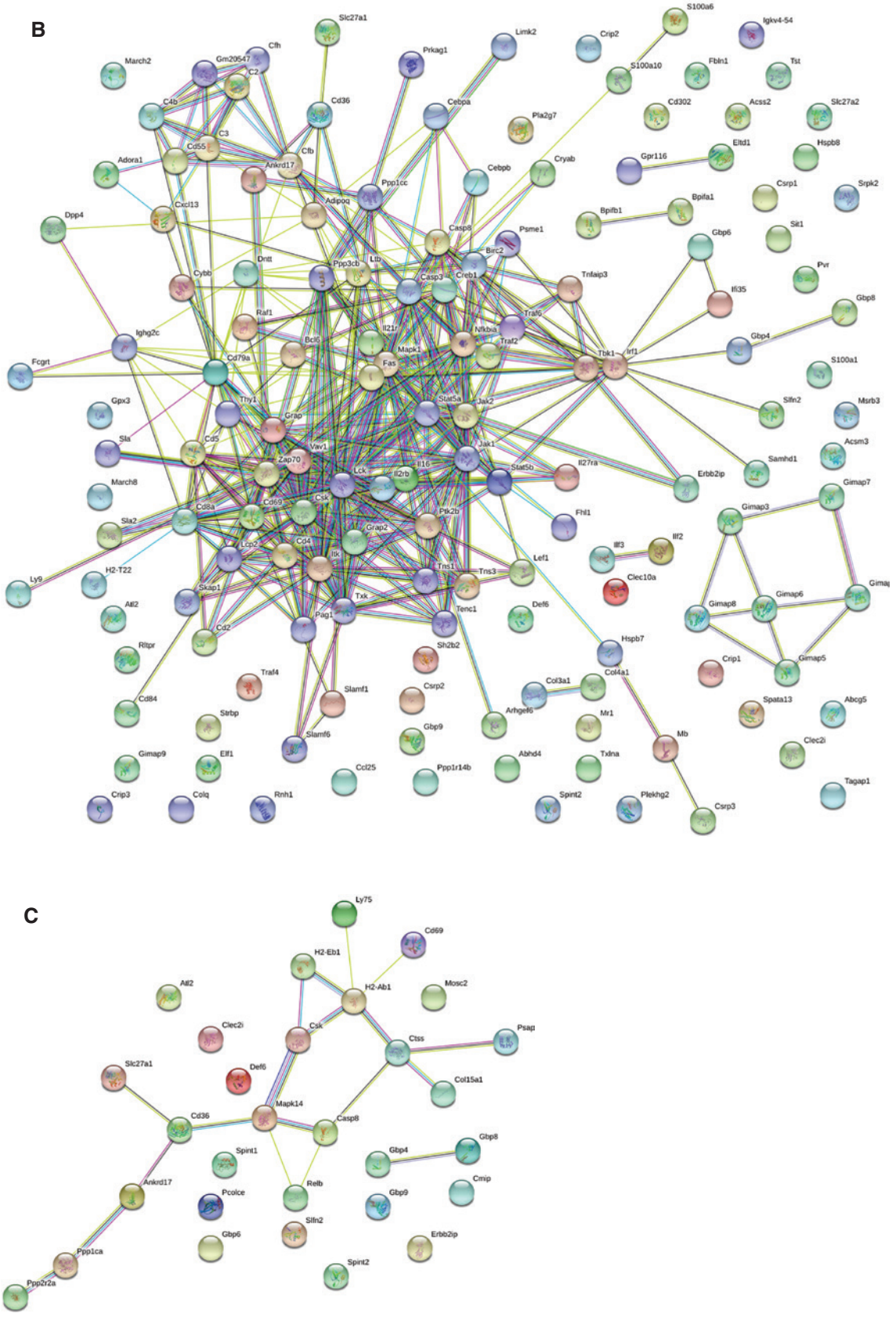


Figure 1: A gene co-expression network of immune related DEGs.
(A) A gene co-expression network between AL-CCR immune-related DEGs. (B) A gene co-expression network between CCR-ICR immune-related DEGs. (C) A gene co-expression network between AL-ICR immune-related DEGs.

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Table 6: Information of co-expression network analysis.

Results of network analysis	AL-CCR	CCR-ICR	AL-ICR
Number of nodes	167	152	30
Number of edges	602	447	19
Average node degree	7.21	5.88	1.27
PPI enrichment p-value	1.0E-16	1.0E-16	2.32E-07

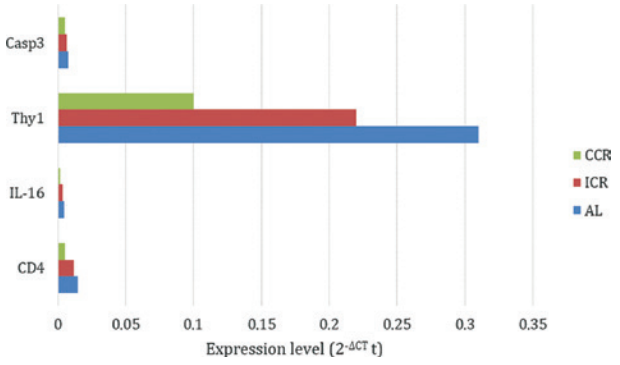


Figure 2: qPCR validation of showing the expression levels of Casp3, Thy1, IL-16 and CD4 genes in CCR, ICR and AL fed groups.

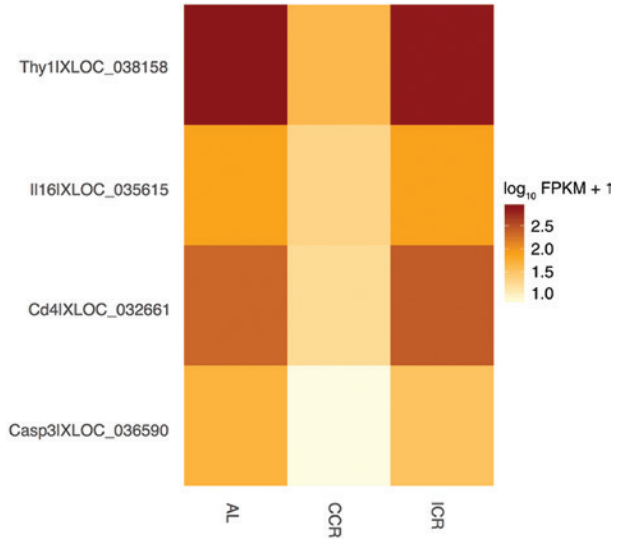


Figure 3: Heat map showing the expression profiles of Casp3, Thy1, IL-16 and CD4 genes in the CCR, AL, and ICR groups revealed by RNA-seq.

2821, 2825, 445 significantly DEGs between the fed groups AL-CCR, CCR-ICR, AL-ICR, respectively. These DEGs were annotated into molecular function, cellular component and biological process GO terms. DEGs in "immune system process (GO:0002376)" GO term were obtained for fed groups. According to these results, 188, 36, 176 differential immune-related genes were determined between AL-CCR, AL-ICR, CCR-ICR fed groups, respectively. These results show that CR and/or the types of calorie consumption have a great effect on immune system. Compared to AL group, although only 15% of CR was applied to CCR fed group, 188 significantly immune-related DEGs were shown between AL and CCR fed groups. Besides, 36 significantly immune-related DEGs were obtained between AL and ICR groups. When the feeding cycle considered, ICR group had 2 weeks of CR (60%) in total of two complete feeding cycles compared to AL. This might suggest that most of the genes were not instantly affected from restriction or they were recovered immediately due to AL feeding part of the cycle. The number of immune-related DEGs were higher in between AL-CCR and CCR-ICR fed groups, than between AL-ICR fed groups. This shows that the expression of immune system genes were regulated up or down by feeding. Moreover the number of up or down regulated genes was more due to chronic but mild feeding restriction compared to severe and acute application.

In this study, *Thy1*, *IL-16*, *CD4* and *Casp3* which play important roles in immune response have been reported as determining expression level in RNA-seq data and qPCR. Expression levels of these genes reduce in CCR fed. Also, many studies have reported decreased level of *Thy1*, *IL-16*, *CD4* and *Casp3* with CR [35–37]. Because, CR is an intervention that dramatically decreases fat mass, helps prevents age-associated diseases, and prolongs lifespan [38]. So, CR decreases disease-associated increases immune genes mRNA levels [39]. Our results showed that the expression level of *Thy1*, *IL-16*, *CD4* and *Casp3* genes in the thymus tissue reduced with CR. These results indicate that application of short-term CR during early age may affect development of immune function positively and *Casp3*, *Thy1*, *IL-16* and *CD4* gens may play important roles in this process.

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Conflicts of interest statement: The authors declare that they have no conflicts of interest.

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