Altered Cd8+ T lymphocyte Response Triggered by Arginase 1: Implication for Fatigue Intensification during Localized Radiation Therapy in Prostate Cancer Patients

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Abstract

Fatigue, the most common side effect of cancer treatments, is observed to intensify during external-beam radiation therapy (EBRT). The underlying molecular mechanisms remain unclear. This study investigated the differentially expressed genes/proteins and their association with fatigue intensification during EBRT.

Fatigue scores measured by FACT-F and peripheral blood were collected prior to treatment (baseline D_0), at midpoint (days 19–21, D21) and endpoint (days 38–42, D42) from men (n=30) with non-metastatic prostate cancer undergoing EBRT. RNA extracted from peripheral blood was used for gene expression analysis. Plasma arginase I and arginine were examined using ELISA and liquid chromatography–tandem mass spectrometry. Differences in fatigue scores, gene and protein expression between times points following EBRT were analyzed by one way ANOVA followed by Post Hoc t-test.

Fatigue scores decreased significantly from baseline (44.6 ± 8.1) to midpoint (37.3 ± 10.6, p=0.000, low scores indicating high fatigue) and to endpoint (37.4 ± 10.1, p=0.001) during EBRT. *ARG1* (encoding arginase type 1) was significantly up regulated from baseline to midpoint of EBRT (fold change =2.41, p<0.05) whereas genes associated with the adaptive immune functional pathway (*CD28, CD27, CCR7, CD3D, CD8A and HLA-DOB*) were significantly downregulated >2-fold between the study time points. The changes in gene expression were associated with patient reported fatigue intensity. Moreover, the upregulation of *ARG1* was negatively correlated with the absolute lymphocyte count (R^2=0.561, p=0.01) only in the high level of fatigue group (n=17) during EBRT.

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Increased *ARG1* expression is known to result in arginine deficiency, which leads to immunosuppression by impairing lymphocyte proliferation and activation. EBRT-induced *ARG1* upregulation may play an essential role in fatigue intensification via the arginine deficiency and suppression of T-cell proliferation pathways. These findings may provide novel insights into the molecular-genetic mechanisms underlying the development and intensification of cancer treatment-related fatigue.

Keywords

Cancer-related fatigue (CRF); Prostate cancer; External beam radiation therapy (EBRT); Lymphocyte; Gene expression

Background

Cancer related fatigue (CRF) is defined as a distressing and persistent subjective feeling of tiredness and exhaustion that is not proportionate to the level of activity or energy exerted, nor completely relieved by sleep and rest [1–4]. It is the most common side effect of cancer treatment including chemotherapy, radiation therapy (RT), or selected biologic response modifiers [5]. Cancer related fatigue affects more than 80% of cancer patients during treatment [6], and nearly 40% of cancer survivors [7], which negatively impacts the health-related quality of life (QOL) of these individuals [8]. Four percent of these patients experienced severe fatigue up to 5 years post cancer treatment [9].

RT for non-metastatic cancer, especially with the use of modulated radiation therapy such as external beam radiation therapy (EBRT) has increased cure and survival rates of patients [10]. RT can however result in numerous side effects, specifically side effects related to targeted organs, including urinary, bowel, and sexual dysfunctions, as well as fatigue in men with non-metastatic prostate cancer receiving EBRT in the pelvic area. [11]. In fact, 71% of these men report clinically significant fatigue while receiving RT [12]. Unlike fatigue related to chemotherapy, which reaches a peak in the days following treatment and then decreases until the next treatment, the fatigue induced by RT, even peripherally-targeted RT often increases gradually in intensity over the course of treatment [13]. There is no U.S. Food and Drug Administration (FDA) approved pharmacological intervention for CRF. During active treatment, the most current National Comprehensive Cancer Network (NCCN) clinical practice guidelines recommended the use of methylphenidate (psychostimulants) for the pharmacological intervention for cancer related fatigue [14]. However, a systematic review and meta-analysis reported the inconsistent effectiveness of methylphenidate on CRF [15]. One possible explanation of this inconsistency and ineffectiveness of treatment for CRF may be due to lack of complete understanding of CRF etiology and mechanisms underlying its development.

Few studies have reported the associations of CRF with impairment in immune function, especially related to T lymphocyte activities. However, the results are inconsistent. In one study, fatigued breast cancer survivors had significantly higher circulating T-cell subsets (CD3+ T lymphocytes, CD4+ T lymphocytes, and CD3+/CD56+ effector T lymphocytes) than non-fatigued breast cancer survivors [16]. Another study conducted by the same

research group found that fatigued breast cancer survivors had lower levels of circulating effector T-cells (CD3+/CD69+) [17]. Although these two studies suggested the role of T-cell mediated inflammation in the development of CRF via cytokine signaling pathways [16,17], further study exploring the association of T-cell alteration, inflammation, and the development of CRF is needed. Particularly, defining the molecular-genetic mechanisms underlying the initiation, intensification, and progression of CRF is critical to developing efficient preventive and treatment strategies. Not only are these strategies necessary to enhance functional status and health-related QOL of patients during cancer therapy, but also during survivorship. Therefore, this study investigated the differential expression of genes and proteins associated with T lymphocyte proliferation, function and their reciprocal associations with self-reported fatigue intensification in men receiving EBRT for nonmetastatic prostate cancer.

Materials and Methods

Subjects, timeline of clinical procedures and sample collection

Subjects with non-metastatic prostate cancer scheduled to receive EBRT were enrolled under an actively recruiting protocol NCT01143467. The protocol was approved by the Institutional Review Board of the National Institutes of Health (NIH), Bethesda, Maryland, USA. Recruitment and data collection were conducted from May 2009 to December 2012 at the Hatfield Clinical Research Center, NIH. Informed consent was obtained from all participants. Patients were excluded if they had any progressive disease causing fatigue; psychiatric disorder within five years; uncorrected hypothyroidism or anemia; second malignancies; or were taking sedatives, steroids, or non-steroidal anti-inflammatory agents.

Fatigue and peripheral blood were collected at baseline (prior to EBRT, D0), midpoint (days 19–21, D21) and endpoint (days 38–42, D 42) of EBRT, when fatigue intensification gradually increased in our previous study [2]. Fatigue intensity was measured using the Functional Assessment of Cancer Therapy-Fatigue subscale (FACT-F), a 13-item self-report questionnaire.

The FACT-F is a frequently used, validated, reliable, and stand-alone measure of fatigue in cancer therapy with coefficient alphas greater than 90 [18,19]. The scores on the instrument range from 0–52, with higher scores indicating less fatigue. In the general population, the mean FACT-F score was 43.6 ± 9.4, whereas in nonanemic cancer patients, it was 40.0 ± 9.8 [18]. A change of 3-point or higher in FACT-F scores is commonly accepted as representing clinically significant and meaningful change in fatigue [19]. All participants were also evaluated using the Hamilton Depression Rating Scale (HAM-D) to exclude subjects with depression. The HAM-D is a 21-item, clinician-rated paper questionnaire with good internal reliability (α=0.81 to 0.98) [20].

Peripheral blood was collected from each subject at each time point to obtain a complete blood count (CBC) and RNA gene analysis using EDTA and PAXgene blood RNA tubes (Qiagen, Frederick, MD, USA), respectively. CBC was analyzed by the NIH Department of Laboratory Medicine using standard hospital procedure. All other samples were stored at −80°C until RNA extraction was performed to reduce intraassay variability. Total RNA was

isolated per manufacturer's protocol. RNA concentration, purity, and integrity were determined using the NanoDrop ((ND-1000; Wilmington, Delaware) and Experion systems (BioRad, Hercules, California), respectively.

Gene expression analysis using Affymetrix microarray

In the study, 90 GeneChips Human Genome U133 Plus 2.0 were used (n=10 at each time point following EBRT). All microarray chips were processed in the same laboratory following standard operating protocols to minimize nonbiological technical bias. A total of 100 ng of purified RNA was used to reverse-transcribe into cDNA, which was used for the synthesis of biotin-labeled cRNA. Subsequently, 15 μg of fragmented cRNA was used to hybridize each chip at 45°C for 16 hours in a GeneChip hybridization oven at 60 rpm. Affymetrix GeneChip Command Console (AGCC, 3.0 V) was used to scan the images and gather raw data. Microarray quality control (QC) was evaluated for each Affymetrix array. Raw data (Affymetrix. CEL files) were imported into Partek® Genomic Suite™ (Partek Inc., St. Louis, MO). Probe summarization and probe-set normalization were performed using Robust Multi-Chip Average (RMA), which includes background correction, quartile normalization, $\log_2$ transformation, and median polish probe set summarization. Principal Component Analysis (PCA) was used to detect outliers. Differences in gene expression between study time points during EBRT were analyzed using repeated measures multiway ANOVA followed by post hoc pairwise comparison to determine the differential gene expression at different time points during EBRT. Differentially expressed genes were identified by at least a 2-fold change in either direction with the p value corrected for false discovery rate (FDR of 0.05). Batch effects were controlled in the analysis by including the scanned dates in the ANOVA model. Biological pathway analysis was performed using Pathway Enrichment.

Verification by quantitative real-time PCR (qRT-PCR)

Differentially expressed genes that were significantly correlated at $r>0.50$ and $p<0.05$ with FACT-F scores were selected for validation using the same RNA samples used in the microarray analysis. Quantitative real time PCR (qRT-PCR) was performed by the Laboratory of Molecular Technology at Frederick National Laboratory for Cancer Research (SAIC-Frederick, Maryland). All reagents used in qRT-PCR were purchased from Applied Biosystems (Foster City, CA). Briefly, 50 ng of RNA in 5 ul of DNase-treated RNA plus 5 μl master mix in 10 μl reaction was used to synthesize cDNA using an ABI High Capacity cDNA reverse transcription kit (ABI 4374967). PCR was performed with Pool TaqMan assays from 20X to 0.2X, including two other genes *RNase P* and *GAPDH*. Complementary DNA template was synthesized using ABI PreAmp master mix (4391128): 5.0 μl 2X master mix + 2.5 μl pooled assays + 2.5 μl RT products, 15 cycles. DNA suspension buffer (20 μl) was added to each reaction and used 2 μl for 5 μl TaqMan reactions with *GAPDH* and *RNase P*. Ct ranged from 7–15. Quantification of gene expression was performed using Fluidigm 96.96 dynamic gene expression array. Data were exported and compiled with Excel.

Plasma arginase I and arginine detection

Concentrations of arginase I and arginine were examined using plasma collected in EDTA tubes from the same subjects in the microarray analysis at all study time points. The plasma samples were separated from whole blood and stored at −80°C. Frozen plasma samples were thawed on ice and centrifuged at 1,000 xg for ten minutes at 4°C. ELISA was performed using 20–50 μl of nonediluted plasma samples. Protein confirmation was tested in triplicate. Each plate was read by VICTOR3 at 450 nm. Arginase I was measured by ELISA (Hycult Biotech, Plymouth Meeting, PA) following the manufacturer's instructions.

Arginine in the plasma was extracted by protein precipitation with 5% TCA containing 20 uM 13C6-arginine (internal standard). The supernatant was analyzed by liquid chromatography – tandem mass spectrometry (LC-MS/MS) with electrospray ionization. Arginine concentrations in the samples were determined from the linear regression curve (1–250 uM) constructed by plotting the peak area ratios against the standard concentrations. The reference limits for plasma L-arginine were established, using a stable isotope based LC-MS/ MS method, at the 2.5^{th} and 97.5th percentiles to be 41.0 μmol/L (95% CI=39.5–42.5 μmol/L) and 114 μmol/L (95% CI=112–115 μmol/L), respectively [21].

Data analyses

As previously described [2,22], descriptive statistics were performed to identify the participants' demographic characteristics. The differences in fatigue intensity, gene and protein expressions at different time points following EBRT were analyzed using one way repeated measures ANOVA followed by post-hoc t-test to compare the differences between control at baseline (D0) to midpoint (D21) and endpoint (D42) during EBRT. The association among the gene expression of interest and the association between the gene expression and patient reported fatigue scores were examined using Pearson correlation. All statistical analyses were conducted using SPSS software version 21 (IBM Corporation, Armonk, New York).

Results

Study population, demographics and fatigue scores

A total of 30 men with non-metastatic prostate cancer (73% Caucasian, 23% African American and 3% other race) between 49–81 years old were included in this study. The demographic and clinical characteristics of the sample are shown in (Table 1). None of the participants reached the cutoff score for depression as measured by the HAM-D either at baseline (mean=1.13 ± 1.76, range 0–8) or at midpoint of EBRT (mean=2.17 ± 3.38, range 0–13) or endpoint (mean=1.37 ± 1.60, range 0–8). Depression levels in the three time points were not statistically significant. Of the 30 subjects, 87% (26/30) received a total dose of 75.6 Gray with EBRT, while the remaining 13% (4/30) received a total dose of 68.4 Gray. Fatigue scores, as measured by FACT-F (low scores indicating high fatigue), significantly decreased from baseline (mean=44.5 ± 8.1) to midpoint (mean=37.3 ± 10.6, p=0.000) and from baseline to endpoint (mean=37.4 ± 10.1, p=0.001) following EBRT. There was no significant difference of fatigue level between midpoint and endpoint of EBRT (Figure 1A), p=0.915. To verify the outcomes of FACT-F assessment, fatigue scores were further

evaluated using the Patient Reported Outcomes Measurement Information System fatigue (PROMIS-F) scale, 7 items, higher scores represent higher levels of fatigue, (Figure 1B) in the same patients during EBRT. As shown in (Figure 1), patient-reported fatigue intensities were peaked at midpoint (D21) and there was no significant difference in terms of fatigue scores between midpoint and endpoint. These findings indicate that in these non-metastatic prostate cancer patients undergoing EBRT, fatigue developed and its intensity reached a peak level at the midpoint of EBRT and maintained near its peak level at completion of EBRT. Thus, to investigate the differential gene expression and the possible biological pathway underlying fatigue intensification during EBRT, we then focused on comparing the differences between baseline (D0) and midpoint (D21) in the following results and discussion sections.

Gene expression profile and regulatory networks of fatigue intensification

In order to clearly describe the changes in gene expression at the initial fatigue intensification during EBRT, the fold changes in gene expression where compared from D_0 prior to EBRT to D21 following EBRT were acquired by use of microarray gene analysis. Compared to D0, there were 327 transcripts with over a 2-fold change in expression at D21 (FDR adjusted $p<0.05$, n=30). The top 10 upregulated and downregulated genes are listed in Table 2. The differentially expressed genes were then analyzed for their associations with canonical pathways. The top three canonical pathways were the T-cell receptor signaling ($p=3.48 \times 10^{12}$), the calciuminduced T lymphocyte apoptosis ($p=1.42 \times 10^{11}$), and the iCOS-iCOSL signaling in T helper cells ($p=8.35 \times 10^{11}$) (Table 3).

The specific differentially expressed genes of interest that were associated with the top 3 canonical pathways were the upregulation of *ARG1* (encoding arginase type 1, fold change =2.41, adjusted $p<0.001$), *CA1* (carbonic anhydrase 1, fold change =3.33, adjusted $p<0.001$), and *XK* (X-linked Kx blood group, fold change =2.62, adjusted $p<0.001$), as well as the down regulation of *CD8A* (encoding the CD8 alpha chain, fold change =−2.17, adjusted $p<0.001$), *CD27* (encoding TNF-receptor superfamily, fold change =−2.11, adjusted $p<0.001$), *CD28* (encoding CD28 molecule, fold change =−2.11, adjusted $p<0.001$), and *CCR7* (chemokine [C-C motif] receptor 7, fold change =−4.27, adjusted $p<0.001$) during the initial fatigue intensification (Table 3). In a parallel ongoing animal model of fatigue-induced by radiation, similar gene expression patterns were found (Table 4).

Verification of changes in gene expression by qRT-PCR

The changes in expression of the genes of interest selected from the microarray findings were further verified using qRT-PCR. *ARG1* was significantly upregulated at D21 of EBRT compared to D0 (Figure 2); (1.72-fold, p=0.017, Kruskal-Wallis One Way Analysis of Variance on Ranks followed by post hoc Tukey - test). In contrast, the expressions of *CD8A, CD27, CD28,* and *CCR7*, were significantly downregulated ($p<0.05$) at D21 of EBRT (Figure 2), *CD8A* 1.98-fold, *CD27* 1.94-fold, *CD28* 1.74-fold, and *CCR7* 1.95-fold) compared to baseline.

Correlation of the changes in the expression genes of interest related to fatigue and their association with fatigue intensity during EBRT

To further examine whether these genes of interest encoding T-cell signaling pathway have a similar expression profile and whether they participate in a common or intersecting biological pathway in the fatigue intensification during EBRT, the Pearson correlation coefficients were calculated among these genes. As shown in Table 5, the upregulation of *ARG1* was highly correlated with the changes in the gene expressions of CCR7, *CD27, CD28, CD8A, XK and CA1.* Moreover, there were significant correlations among the changes in expression of the genes related to T-cell proliferation *CCR7, CD27, CD28* and *CD8A* during EBRT.

More interestingly, the upregulation of *ARG1* gene expressions following EBRT was positively correlated with the reported fatigue scores patients as measured by the PROMIS-F (higher score indicating higher level of fatigue) whereas the upregulation of the gene expression of *CCR7*, *CD27* and *CD28* was negatively correlated to the patient reported fatigue intensity (Table 6).

Correlation between *ARG1* gene expression and lymphocyte count

Since the pathway analysis indicated that these specific differentially expressed genes of interest are mainly associated with the T lymphocyte signaling pathway (p=3.48×10^{12}) during EBRT, we further examined whether the upregulation of *ARG1* is associated with the changes in lymphocyte account during EBRT. As shown in Figure 3, EBRT-related upregulation of *ARG1* was negatively correlated with the change in absolute lymphocyte count (Figure 3A), r=0.47, p=0.01) during EBRT. Further analysis between the high versus low fatigue groups (high fatigue, >3-point change in FACT-F score from baseline to midpoint of EBRT), the association of *ARG1* expression and change in absolute lymphocyte count was only observed in the high fatigue group (Figure 3B), r=−0.56) but not in the low fatigue group (r=−0.003).

Differential expression levels of arginase I and arginine in plasma between the high and low fatigue subjects

Finally, the arginase I and arginine levels in plasma were examined based on their metabolic pathway. As shown in (Figure 4), the arginase level in plasma showed an increased trend in high fatigue (HF) group at midpoint (9.4 ± 8.8) compared to baseline (7.7 ± 2.4); whereas, in the low fatigue (LF) group, arginase level in plasma significantly declined at midpoint (6.9 ± 2.4) from baseline (8.5 ± 4.6, p=0.02). For arginine, its level in plasma decreased at midpoint (117.5 ± 26.1) compared to baseline (125.8 ± 30.7) in the HF group and remained unchanged at midpoint (121.7 ± 28.1) from baseline (121.3 ± 26.8, p= 0.82) in the LF group. However, the changes in arginase and arginine in the HF group did not reach statistical significance, possibly due to the large inter-individual variation and limited sample size.

Discussion

The principal novel finding from this study is the increase in the expression of arginase 1 ARG1 that is correlated with the decrease in the absolute lymphocyte count in the prostate

cancer patients who developed significant fatigue during EBRT. Moreover, the highly significant correlation between expression of ARG1 and the expression of T-cell proliferation markers of *CD8, CD28, CD27,* and *CCR7* indicate that *ARG1* mRNA levels were mainly expressed by T lymphocytes. This latter finding has been previously reported [23] and these protein encoding genes are noted to be highly expressed in less-differentiated T cells [24,25]. Although the changes did not reach statistical significance, there was a trend for HF subjects to show an increase in arginase and a decrease in arginine in plasma at midpoint when compared to baseline. Our findings are consistent with previous reports that T lymphocytes are suppressed by depletion of L-arginine through increased expression and activity of arginase 1 [26]. Moreover, our findings indicate that the reciprocal interplay of arginine metabolism and T lymphocyte activity with fatigue development and intensification during localized RT in non-metastatic prostate cancer patients. We hypothesize that fatigue intensification during EBRT in this clinical population may be associated with: (1) EBRT-related changes in the arginine metabolism pathway that trigger the arginase 1 activity, and (2) the subsequent reciprocal interplay of arginine deficiency and T cell receptor signaling downregulation causing suppression of T cell mediated immune response (Figure 5).

Immune dysfunction and fatigue

It is well known that adaptive immune response including T helper (TH) and cytotoxic T lymphocytes (CTL) occurs during the early stage of tumor growth and results in the development of CD8+ T cells reactive to tumors [27]. The CD8 antigen is found in all cytotoxic T lymphocytes (CD8+ T cells) and acts as the key player in antitumor immune response by antigen recognition. However, naïve CD8+ T cells need a co-stimulatory signal to activate and maintain their optimal activation [28]. CD27, CD28 and CCR7, which are highly expressed on naive CD8+ T cells [25], are essential to co-stimulate CD8+ T cell activation, proliferation, survival, and differentiation [29–31].

The CD8+T cells have consistently been shown to be decreased in patients with chronic fatigue [32–34]. One of the differences between patients with chronic fatigue and healthy controls was the striking down-regulation of CD+8 T cells with significantly lower activation, proliferative response and frequency of effector memory cells in individuals with chronic fatigue [24]. Furthermore, the CD27 receptor transduces signals that lead to the activation of NF-kappaB and MAPK/JNK pathways, which are also associated with the development of chronic fatigue pathways [35–38]. The current findings of decreased expression of *CD8, CD28 CD27, CCR7* and *CD3* during EBRT suggest that a CD+8 T-cell suppressed responses may play a key role in the initial development of fatigue in cancer patients undergoing radiation therapy (RT).

Arginine deficiency, cell dysfunction, and immune suppression

One of the mechanisms underlying T lymphocyte cell-mediated fatigue may be related to the activities of L-arginine metabolism enzyme, arginase 1 [39]. Arginase remains a crucial player in host defense; specifically arginase 1, which is selectively expressed in granulocytes (polymorphonuclear leukocytes) where it modulates the immune system through arginine depletion [40]. It was reported that increased arginase 1 (ARG 1) activity in prostate cancer and lung cancer patients is involved in peripheral immunosuppression [41–43]. High

arginase 1 activity depletes L-arginine and the downstream effects of which can inhibit CD8+ T-cells proliferation and activation, or trigger T-cell apoptosis because arginine is required for T-cell receptor formation and proliferation [44–46]. Activated CD8+ T-cells are particularly sensitive to the L-arginine level in their microenvironment [47]. Low or absent L-arginine significantly decreases proliferation of T-cells in vitro and activated T-cells are arrested in the G0-G1 phase of cell cycle; whereas T-cells cultured with L-arginine have their proliferation restored into the S and G2-M phases [48].

The main effect of over expression of arginase 1 on T-cell suppression is caused by L-arginine depletion [23,49] because L-arginine, a semiessential amino acid, plays a central role in immune response through the arginine metabolic pathway [43]. Arginine metabolism through arginase 1 produces urea and ornithine [50]. The latter is a precursor of different products, including polyamines and proline, which play a key role in cell proliferation [51] and wound healing [43]. T lymphocytes depend on arginine for multiple key biological processes, including proliferation, expression of the TCR complex and the ζ-chain peptide, and the development of memory T-cells [52]. With arginine deprivation, there is a progressive reduction (to ~25% of basal levels) in the number of T-cell receptors on the cell membrane, as observed in certain cancers and after surgery or trauma [53,54]. A few hours after injury, a decrease in circulating arginine and an increase in accumulating arginase 1 expressed in immature myeloid cells further indicate that the arginine deficit is largely influenced by liberated arginase, rather than lack of arginine intake [52]. It is worth mentioning that the release of cytokines (IL-4, IL-10 and IL-13), catecholamines, and prostaglandin E2 (PGE2) by malignant tumors and adjacent tissue may also lead to arginase accumulation that enables synergism to deplete serum and cellular arginine, and ultimately lead to the depletion of arginine and result in impairment of T-cell proliferative response [55].

The current findings show the differential expression of *ARG1* gene, increased arginase 1 level, and decreased arginine concentration in plasma of prostate cancer patients developed fatigue during EBRT. Furthermore, the decreased lymphocyte count in HF subjects during EBRT suggests the interplay between arginine metabolic pathway and CD8+ T-cell function as the potential mechanisms to explain fatigue intensification in these subjects. We hypothesize that the arginine-immune suppression-fatigue pathway shown in (Figure 5) triggered by EBRT may explain how EBRT causes an over expression of arginase, and how arginine depletion causes suppression of T-cell function and translates into a change in behavior, such as fatigue intensification.

Clinical implications

Existing arginase inhibitors or exogenous arginine administration have been shown to re-establish T-cell proliferation [56,57]. While several caveats should be taken into consideration when evaluating the current findings, we discuss the potential strategies for clinical consideration to relieve the debilitating fatigue associated with cancer treatment.

Blockade of arginase expression and upregulation: Arginase inhibitors N-Hydroxy-nor-l-Arg (NorNOHA) and N-Hydroxy-l-Arg (NOHA) have been shown to

partially prevent the reduction of l-arginine in culture T-cells and in an animal model [58]. Therefore, Arg-1 inhibitors may represent a target for development of new therapies to alleviate fatigue related to cancer and cancer treatment.

Arginine supplementations: increasing arginine level by exogenous L-arginine or dietary use of arginine supplementations have been shown to improve immune response in surgical patients. Citrulline, an arginine precursor, has been shown to counter high arginase activity [59]. Because tumor cells and PMNs release arginase through the COX-PGE E2 pathway [52,60], cyclooxygenase-2 (COX-2) inhibitors (e.g., celecoxib and sc-59125) could be used to reduce or prevent fatigue since these agents have been shown to prevent arginase 1 over expression and inhibit tumor growth [61].

Conclusions

Our current findings indicate that increased arginase 1 expression at the transcriptional level may play a critical role in fatigue intensification during EBRT, possibly through arginine deficiency-associated down-regulation on T-cell proliferation. These findings provide evidence at the transcriptional level of possible biologic correlates of cancer treatment-related fatigue, which may provide important insights into the molecular-genetic mechanisms that could be used in the development of new strategies for its prevention and treatment.

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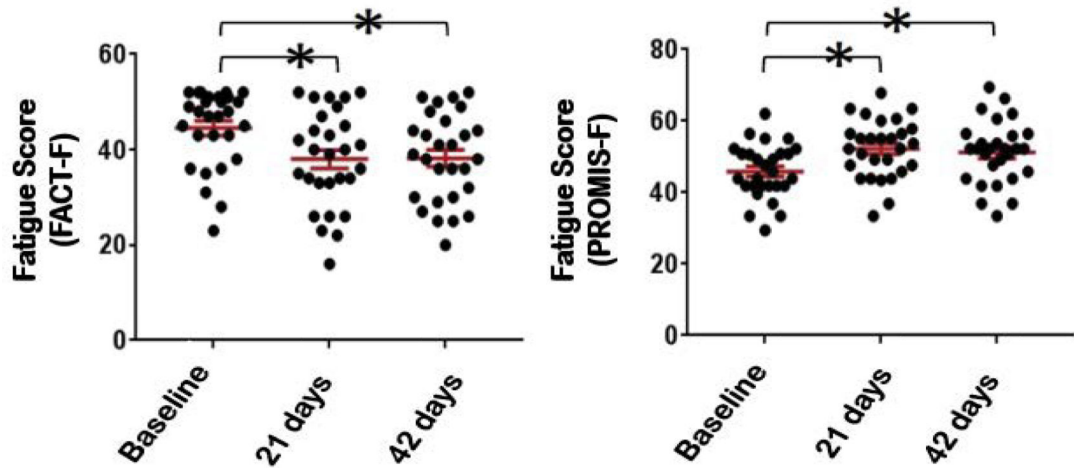
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Figure 1:

Patient reported fatigue scores during external beam radiation therapy (EBRT). Changes in fatigue scores following EBRT in non-metastatic prostate cancer patients were assessed at midpoint of EBRT (D21) and endpoint of EBRT (D42) compared to baseline (D0). A. Fatigue score was measured by Functional Assessment of cancer therapy (FACT-F, 13 items, higher scores represent lower levels of Fatigue). B. Fatigue score was measured by the Patient Reported Outcomes Measurement Information System fatigue (PROMIS-F, 7 items, higher scores represent higher level of fatigue). **, $p < 0.001$, *, $p < 0.05$ compared to baseline (Friedman Repeated Measures Analysis of Variance on Ranks followed by Post hoc Tukey Test). There was no significant difference in fatigue scores between midpoint and endpoint as EBRT measured by FACT-F or PROMIS-F.
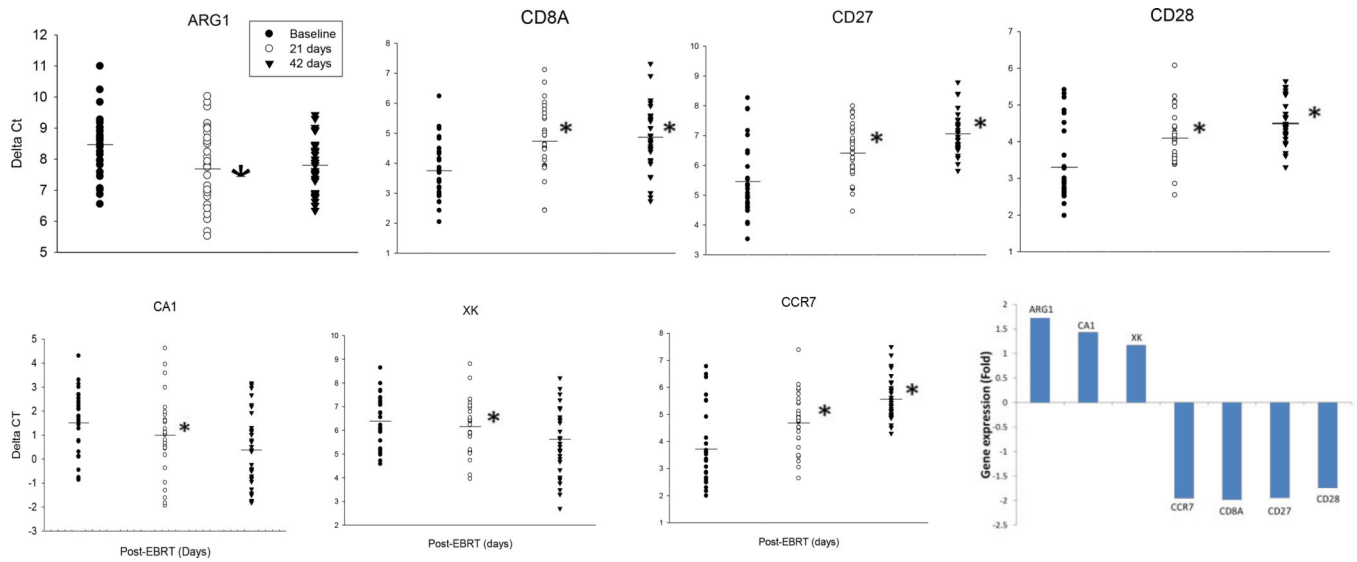
Figure 2:
Changes in gene expression during external beam radiation therapy (EBRT) in non-metastatic prostate cancer patients at midpoint (D21) and endpoint (D42) compared to baseline (D0) as assessed by qRT-PCR. The gene expression level is expressed as the average threshold cycle after normalization using GAPDH expression (Average Delt Ct). The bars represent mean; *p<0.05 significant difference from the baseline (D0) (Kruskal-Wallis one-way ANOVA followed by Tukey test).
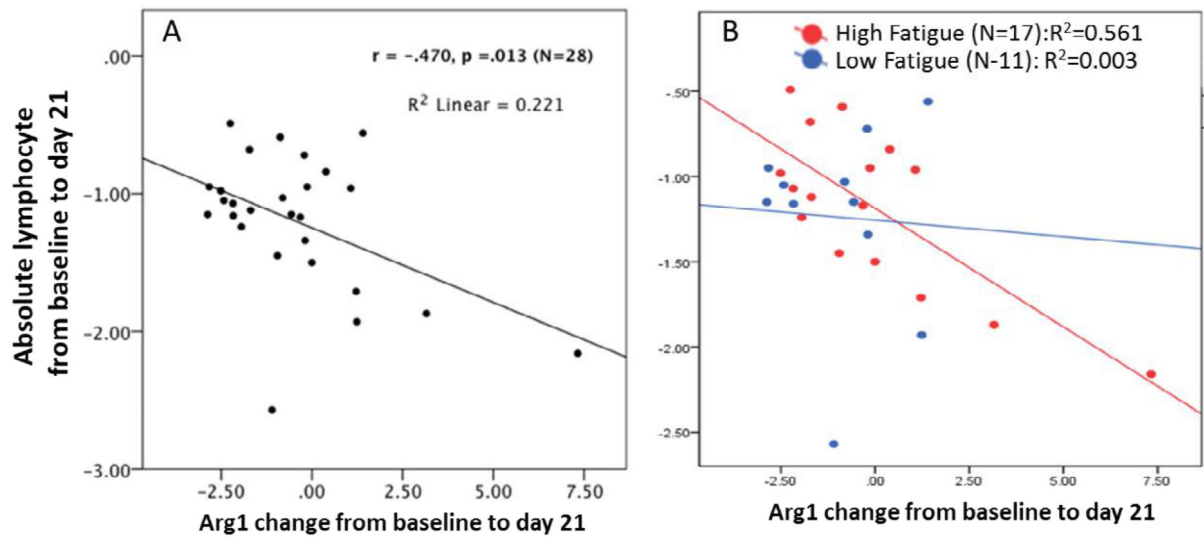
Figure 3:
Correlation between gene expression level of *ARG1* and absolute lymphocyte count atmidpoint of EBRT (D19–21) from fatigued prostate cancer patients during ERBT (3A). Further analysis indicates that the significant correlation was only observed in the high fatigued subjects (3B).

Figure 4:
Different expression of arginase and arginine in plasma at midpoint of EBRT (D19–21) from baseline between high and low fatigue groups. * $p < 0.05$, Wilcoxon Mann-Whitney test.

Figure 5:

Schematic representation of ARG1-arginine-immune suppression-fatigue pathway. EBRT induces the increase in arginase 1 at transcriptional level (ARG1) or the increase in arginase accumulation; arginase 1 is the k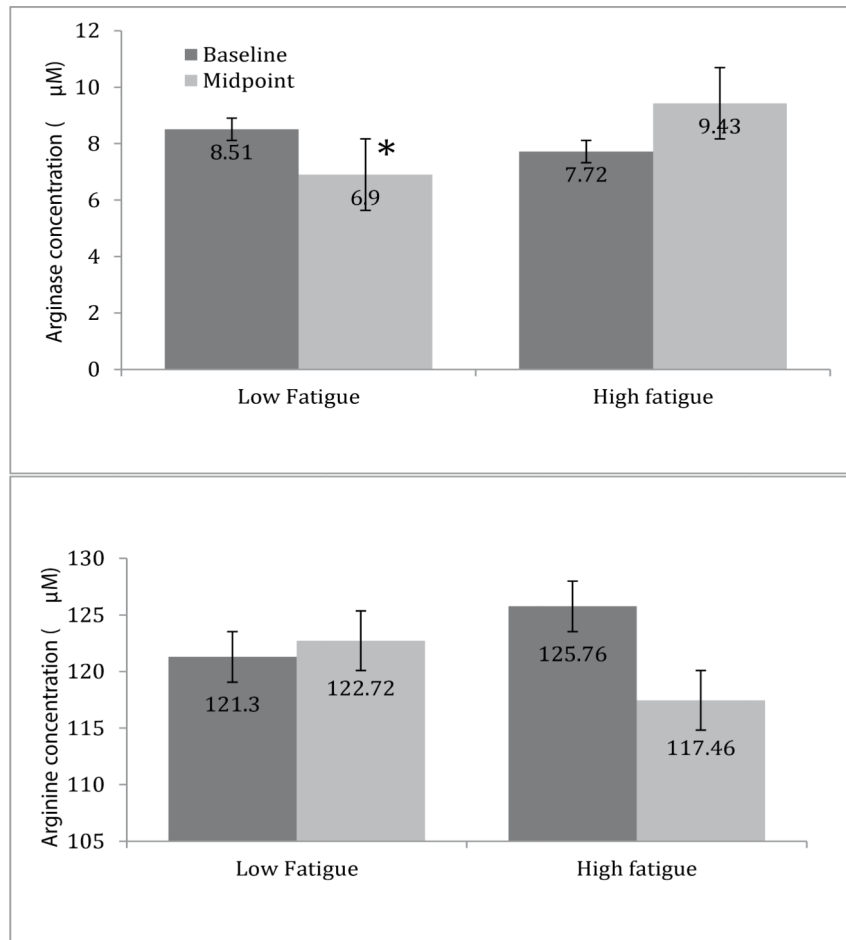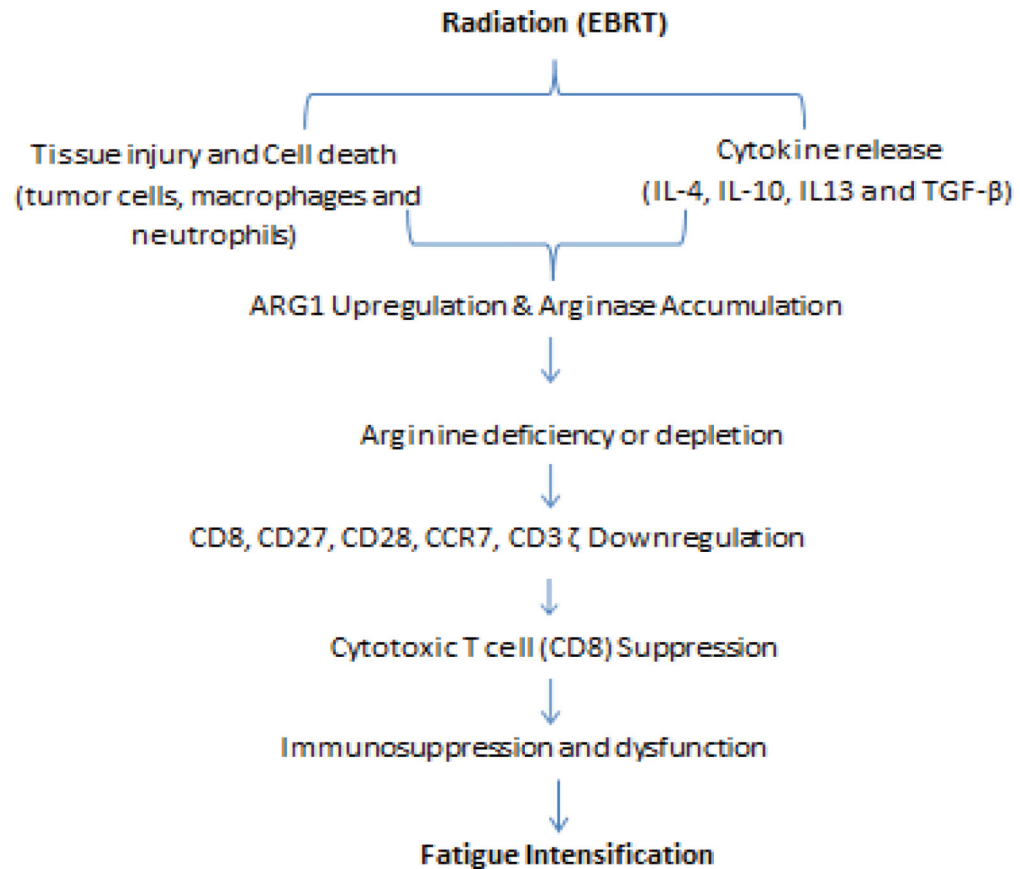ey enzyme to catalyzes arginine and can lead to deficiency or depletion of arginine at cellular or plasma level. The latter results subsequently in the suppression of T lymphocytes by down-regulation of the expression CD8, CCR7, CD27, CD28 and CD3, which are essential to lymphocyte activation, differentiation, proliferation and survival. Arginine deficiency induced T lymphocyte suppression plays a central role in immune response associated chronic fatigue syndrome. Therefore, it is reasonable to hypothesize that EBRT-related changes in the arginine metabolism pathway by triggering the arginase 1 activity lead to the subsequent reciprocal interplay of arginine deficiency and suppression of T cell mediated immunity response, which may play the important role in the initial fatigue development and intensification in these cancer patients undergoing radiation therapy.

Table 1:

Demographics and Clinical Characteristics of Sample (N=30).

	Mean		Range	N (%)	Repeated measure ANOVA p value
Age in Years	64.3	7.5	49–81	30 (100)	
Race					
Caucasian				22 (73)	
African-American				7 (23)	
Other				1 (3)	
Clinical T stage					
T1 (a-c)				11 (36.7)	
T2 (a-c)				17 (56.7)	
T3 (a-c)				2 (6.7)	
Gleason score	7.6	0.99	06–09		
BMI	29.5	4.1	22.9–40.2		
Total EBRT Dosage (Gray)					
75.6				26 (87)	
68.4				4 (13)	
PSA Levels (ng/mL)					
Baseline	10.4	10.7	0.08–33.4		
Completion	0.23	0.6	0.01–2.59		
Albumin levels (g/dL)					
Baseline	4.1	0.8	3.6–4.5		
Testosterone (ng/dL)					
Baseline					
TSH (mcIU/ml)					
Baseline	2.26	1.44	0.24–4.15		
Hemoglobin					
Baseline	13.5	1.1	10.9–15.1		
FACT-F Questionnaire					
Baseline	44.57	8.1	23–52		
Midpoint	37.34	10.6	16–52		0.000[a]
Endpoint	37.38	10.1	14–52		0.001
HAM-D Questionnaire					
Baseline	1.13	1.76	0–8		
Midpoint	2.17	3.38	0–13		0.137
Endpoint	1.37	1.67	0–8		

Abbreviations: BMI, body mass index; dL, deciliter; EBRT, external beam radiation therapy; g, gram; mcL, microliter; mL, milliliter; ng, nanogram; PSA, prostate specific antigen; TSH, thyroid stimulating hormone.

[a]*Note:*Repeated measure p value <0.01, Pairwise comparisons showed significant difference in mean between baseline and midpoint of EBRT (p =0.000), and between baseline and endpoint of EBRT (p=0.001). There is no significant difference in mean between midpoint and endpoint of EBRT (p =0.915).

Table 2:

Top differentially expressed genes between baseline and midpoint of EBRT (N = 30).

Probeset ID	Gene symbol	Fold change	P value	Gene Title
Top 10 up regulated genes				
205950_s_at	CA1	3.33	1.22×10^{7}	carbonic anhydrase I
206698_at	XK	2.62	2.09×10^{5}	X-linked Kx blood group (McLeod syndrome)
236081_at	SNCA	2.60	1.04×10^{5}	synuclein, alpha (non A4 component of amyloid precursor)
223669_at	HEMGN	2.46	6.63×10^{6}	ATP-binding cassette, sub-family C (CFTR/MRP), member 13
214407_x_at	GYPB	2.43	8.62×10^{7}	glycophorin B (MNS blood group)
206177_s_at	ARG1	2.41	3.44×10^{5}	arginase, liver
203502_at	BPGM	2.31	5.56×10^{5}	2,3-bisphosphoglycerate mutase
226811_at	FAM46C	2.28	5.27×10^{5}	family with sequence similarity 46, member C
203116_s_at	FECH	2.17	6.54×10^{5}	ferrochelatase
1552583_s_at	ABCC13	2.16	8.54×10^{8}	ATP-binding cassette, sub-family C (CFTR/MRP), member 13, pseudogene
Top 10 down regulated genes				
228592_at	MS4A1	−8.53	1.36×10^{16}	membrane-spanning 4-domains, subfamily A, member 1
212827_at	IGHM	−6.83	3.81×10^{19}	immunoglobulin heavy constant mu
221969_at	PAX5	−4.32	1.51×10^{17}	paired box 5
206337_at	CCR7	−4.25	4.73×10^{14}	chemokine (C-C motif) receptor 7
205267_at	POU2AF1	−4.24	7.21×10^{16}	POU class 2 associating factor 1
236796_at	BACH2	−3.90	5.88×10^{18}	B-cell scaffold protein with ankyrin repeats 1
230245_s_at	LOC283663	−3.85	1.98×10^{18}	hypothetical LOC283663
39318_at	TCL1A	−3.50	2.00×10^{16}	T-cell leukemia/lymphoma 1A
231093_at	FCRL3	−3.45	2.32×10^{12}	Fc receptor-like 3
229513_at	STRBP	−3.32	7.28×10^{18}	spermatid perinuclear RNA binding protein

Table 3:

Top 3 canonical pathways and genes associated with each pathway.

Symbol	Gene name	Probeset ID	p-value	Fold Change
T cell Receptor signaling ($p = 3.48 \times 10^{12}$, Ratio = 14/109 (0.128))				
CAMK4	calcium/calmodulin-dependent protein kinase IV	229029_at	5.09E-10	−2.558
CD28	CD28 molecule	206545_at	1.56E-06	−2.111
CD247	CD247 molecule	210031_at	1.66E-08	−2.330
CD3D	CD3d molecule, delta (CD3-TCR complex)	213539_at	1.58E-06	−2.057
CD3G	CD3g molecule, gamma (CD3-TCR complex)	206804_at	2.37E-07	−2.114
CD8A	CD8a molecule	205758_at	3.16E-06	−2.174
ITK	IL2-inducible T-cell kinase	211339_s_at	9.98E-08	−2.326
LAT	linker for activation of T cells	211005_at	2.19E-10	−2.198
LCK	lymphocyte-specific protein tyrosine kinase	204891_s_at	7.45E-11	−2.536
NFATC2	nuclear factor of activated T-cells, cytoplasmic, calcineurindependent 2	226991_at	4.69E-09	−2.074
PRKCQ	protein kinase C, theta	210038_at	6.59E-09	−2.023
RASGRP1	RAS guanyl releasing protein 1 (calcium and DAG-regulated)	205590_at	1.49E-06	−2.372
RRAS2	related RAS viral (r-ras) oncogene homolog 2	212589_at	3.71E-06	−2.251
TXK	TXK tyrosine kinase	206828_at	9.63E-09	−2.510
Calcium-induced T Lymphocyte Apoptosis ($p = 1.42 \times 10^{11}$, Ratio = 11/71 (0.155))				
CAMK4	calcium/calmodulin-dependent protein kinase IV	229029_at	5.09E-10	−2.558
CD247	CD247 molecule	210031_at	1.66E-08	−2.330
CD3D	CD3d molecule, delta (CD3-TCR complex)	213539_at	1.58E-06	−2.057
CD3G	CD3g molecule, gamma (CD3-TCR complex)	206804_at	2.37E-07	−2.114
HLA-DOB	major histocompatibility complex, class II, DO beta	205671_s_at	8.02E-11	−2.080
ITPR1	inositol 1,4,5-trisphosphate receptor, type 1	240052_at	1.02E-10	−2.288
LCK	lymphocyte-specific protein tyrosine kinase	204891_s_at	7.45E-11	−2.536
NFATC2	nuclear factor of activated T-cells, cytoplasmic, calcineurindependent 2	226991_at	4.69E-09	−2.074
PRKCA	protein kinase C, alpha	213093_at	6.22E-08	−2.316
PRKCH	protein kinase C, eta	218764_at	6.70E-09	−2.216
PRKCQ	protein kinase C, theta	210038_at	6.59E-09	−2.023
iCOS-iCOSL Signaling in T Helper Cells ($p = 8.35 \times 10^{11}$, Ratio = 13/126 (0.103))				
CAMK4	calcium/calmodulin-dependent protein kinase IV	229029_at	5.09E-10	−2.558
CD28	CD28 molecule	206545_at	1.56E-06	−2.111
CD247	CD247 molecule	210031_at	1.66E-08	−2.330
CD3D	CD3d molecule, delta (CD3-TCR complex)	213539_at	1.58E-06	−2.057
CD3G	CD3g molecule, gamma (CD3-TCR complex)	206804_at	2.37E-07	−2.114
HLA-DOB	major histocompatibility complex, class II, DO beta	205671_s_at	8.02E-11	−2.080
ITK	IL2-inducible T-cell kinase	211339_s_at	9.98E-08	−2.326
ITPR1	inositol 1,4,5-trisphosphate receptor, type 1	240052_at	1.02E-10	−2.288

Symbol	Gene name	Probeset ID	p-value	Fold Change
LAT	linker for activation of T cells	211005_at	2.19E-10	−2.198
LCK	lymphocyte-specific protein tyrosine kinase	204891_s_at	7.45E-11	−2.536
NFATC2	nuclear factor of activated T-cells, cytoplasmic, calcineurindependent 2	226991_at	4.69E-09	−2.074
PLEKHA1	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 1	219024_at	6.50E-09	−2.171
PRKCQ	protein kinase C, theta	210038_at	6.59E-09	−2.023

Table 4:

Comparison of changes in gene expression following EBRT in human and mouse.

Probeset ID	Gene Symbol	Fold	p-value	Gene Title
206177_s_at	ARG1	2.41568	0.0000344	arginase, liver
1418847_at	Arg2	3.66503	4.01921E-06	arginase type II
205758_at	CD8A	−2.1743	3.76E-08	CD8a antigen molecule
1451673_at	Cd8a	−2.64612	2.63446E-05	CD8 antigen, alpha chain
1426170_a_at	Cd8b1	−3.54196	1.92496E-06	CD8 antigen, beta chain 1
206545_at	CD28	−2.11077	6.71E-09	CD28 molecule
1437025_at	Cd28	−2.78915	8.28414E-07	CD28 antigen
206150_at	CD27	−2.10736	1.24E-14	CD27 molecule
1452389_at	Cd27	−2.32832	8.33527E-06	CD27 antigen
206337_at	CCR7	−4.26661	4.73E-14	chemokine (C-C motif) receptor 7
1423466_at	Ccr7	−6.26659	1.0981E-09	chemokine (C-C motif) receptor 7
213539_at	CD3D	−2.05128	2.97753E-08	CD3d molecule, delta (CD3-TCR complex)
206804_at	CD3G	−2.09743	3.32498E-09	CD3g molecule, gamma (CD3-TCR complex)
1422828_at	Cd3d	−2.49422	2.25E-05	CD3 antigen, delta polypeptide
1419178_at	Cd3g	−4.40508	3.17E-07	CD3 antigen, gamma polypeptide
220059_at	STAP1	−2.77349	2.74E-15	signal transducing adaptor family member 1
1421098_at	Stap1	−1.99621	0.00011027	signal transducing adaptor family member 1
226218_at	IL7R	−2.87866	9.74E-10	interleukin 7 receptor
1448575_at	Il7r	−4.00297	4.4183E-08	interleukin 7 receptor
212827_at	IGHM	−6.84302	3.81E-19	immunoglobulin heavy constant mu
1427329_a_at	Igh-6	−4.69937	8.32855E-08	immunoglobulin heavy chain 6
1455530_at	Ighv14–2	−16.3121	3.64611E-11	immunoglobulin heavy variable V14–2
1421653_a_at	Igh-2	−10.2349	8.58544E-06	immunoglobulin heavy chain 2 (serum IgA)
231124_x_at	LY9	−2.72628	1.4E-13	lymphocyte antigen 9
1416930_at	Ly6d	−4.76003	5.51E-07	lymphocyte antigen 6 complex, locus D
227646_at	EBF1	−2.61277	1.81E-16	early B-cell factor 1
1416301_a_at	Ebf1	−2.29925	4.28E-05	early B-cell factor 1

Table 5:

Association among gene Arg1, CCR7, CD27, CD28, and CD8A expression (delta Ct) presented as Pearson correlation coefficients (p value).

Gene Symbol	ARG1	CCR7	CD27	CD28	CD8A
ARG1	1	0.93577 (1.31E-41)	0.960055 (1.86E-50)	0.947467 (2.44E-45)	0.93528 (1.82E-41)
CCR7		1	0.991251 (3.47E-79)	0.994166 (6.65E-87)	0.962445 (1.29E-51)
CD27			1	0.993718 (1.71E-85)	0.98082 (2.78E-64)
CD28				1	0.974993 (2.88E-59)
CD8A					1

Table 6:

Correlation between gene expression of interest and fatigue intensity (PROMIS) following RBRT.

Gene name	r value	p value
ARG1	0.2285	0.040192
XK	0.120924	0.282218
CA1	0.227495	0.041102
CCR7	−0.28672	0.009456
CD27	−0.25271	0.022839
CD28	−0.26166	0.018292
CD8A	−0.15419	0.169327