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ORIGINAL ARTICLE

Chemotherapy-induced fatigue is associated with changes in gene expression in the peripheral blood mononuclear cell fraction of patients with locoregional breast cancer

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Abstract

Purpose Chemotherapy-induced fatigue (CIF) is a frequent symptom that impairs patient functioning and quality of life. We aimed to evaluate whether systemic chemotherapy can induce a specific gene expression profile in peripheral blood mononuclear cells (PBMNC) of patients with locoregional breast cancer (LRBC) who develop CIF.

Methods PBMNC were collected from 3 patients who developed CIF before and after their initial cycle of chemotherapy, and RNA-seq was performed in an Ion Torrent™ System. A total of 12.345 transcripts were sequenced, of which 26 were selected out of 71 that had significantly different expression before and after chemotherapy. The RNA-seq results were validated by RT-qPCR in a different group of 28 patients with LRBC who developed CIF after their first cycle of chemotherapy and in six patients who also received chemotherapy but did not develop CIF (controls). We assessed CIF according the BFI and Chalder Questionnaires.

Results We observed a significant increase in expression of DUSP18 and RHOBTB1 and decreased expression of NCAN and RAET1G in patients who developed CIF after chemotherapy. Control patients only exhibited a significant decrease in NCAN expression.

Conclusion CIF induces specific changes in gene expression in the PBMNC of LRBC patients. Some of these changes, such as downregulation of NCAN expression, may reflect direct effects of chemotherapy since they are also observed in the controls. Furthermore, CIF may involve downregulation of skeletal muscle genes (RHOBT1, DUSP18) and immune systems (RAETG1), whereas NCAN downregulation may underlie the adverse cognitive effects of chemotherapy.

Keywords Fatigue · Chemotherapy · Transcriptome · Gene expression

Introduction

Cancer-related fatigue (CRF) is a frequent and multifactorial condition, that is, characterized by a persistent and subjective sense of tiredness related to cancer or its treatment, that is, not relieved by rest [1]. CRF affects more than half of all cancer patients and can be caused by several reversible conditions, such as hypothyroidism, anemia, sleep disturbance, menopausal symptoms, adverse reactions to medication, depression, and anxiety. Cancer treatments, such as chemotherapy, radiation therapy, and new targeted therapies, can also play a role in the genesis of CRF [1–3].

Chemotherapy-induced fatigue (CIF) is a frequent and potentially debilitating side effect of chemotherapy that affects approximately 40% of patients and is severe in approximately

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one fourth of those affected [3, 4]. In addition to anemia, chemotherapy may induce fatigue through several mechanisms, including stimulation of the inflammatory response mediated by changes in cytokine levels, hormonal deregulation, or muscular toxicity [4–6].

Several authors have already described the use of peripheral blood cells as a surrogate material that can be easily obtained to evaluate gene expression in other body tissues, such as the lung [7] and brain [8, 9]. This work aims to better understand the possible molecular basis of these chemotherapy-induced physiological changes by evaluating the gene expression of peripheral blood mononuclear cells (PBMNC) of locoregional breast cancer (LRBC) patients scheduled to receive chemotherapy. Using PBMNC samples from patients who developed fatigue after their first chemotherapy session compared with their own samples obtained before treatment, we were able to identify several genes with significantly different expression between these two-time points. We then selected a group of these genes based on the magnitude of their differential expression and their potential functional roles in important physiological processes, such as inflammation, cellular ion transport, muscular physiology, and cytokine and hormone regulation. We then validated the results in a larger group of LRBC patients.

Methods

Patients

All patients included had LRBC (i.e., initially diagnosed histologically proven breast carcinoma restricted to the breast and/or ipsilateral axillary lymph nodes). Patients whose samples were used in this study participated in a previously published study and, at that time, provided consent for their samples to be used in future studies [10]. Our Institutional IRB also approved the original study as described in [10]. Samples were frozen at − 70 °C until they were used for the analysis reported here. We evaluated fatigue before and after chemotherapy by the BFI [11] and Chalder [12] fatigue questionnaires previously validated in Portuguese [10]. The Chalder scale has 11 items answered on a four-point scale ranging from the asymptomatic ("0") to maximum symptomology ("3"). The respondent's global score can range from 0 to 33. The global score also spans two dimensions—physical fatigue (measured by items 1–7) and psychological fatigue (measured by items 8–11). The one-page BFI has only nine items, with the items measured on 0–10 numeric rating scales. Three items ask patients to rate the severity of their fatigue at its "worst," "usual," and "now" during normal waking hours, with 0 being "no fatigue" and 10 being "fatigue as bad as you can imagine." Six items assess the amount that fatigue has interfered with different aspects of the patient's life during the past 24 h. The interference items are measured on a 0–10 scale, with 0 being "does not interfere" and 10 being "completely interferes" [11].

Total RNA isolation and RNA-seq

Initially, the total transcriptome was sequenced for PBMNC samples collected from three patients who developed CIF before and after their first cycles of chemotherapy. Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, EUA) and treated with DNAseI (Invitrogen) for 15 min at room temperature. Total RNA quantification and integrity were assessed using a BioAnalyzer 2100 (Agilent). RNA-seq was performed in the PGM Ion Torrent™ System using the Ion AmpliSeq™ Transcriptome Human Gene Expression Kit (ThermoFisher Scientific) according to the manufacturer's recommendations. Patients' blood samples were not collected in the same day; however, total RNA was isolated just after blood collection by the same technician, using the same methodology. Furthermore, we included in this study only RNA samples with acceptable RIN (> 7). All samples were delivered to RNA-seq in a single batch and all libraries and sequencing reactions were performed in another lab, but in the same day, by the same technician, using the same reagents lot. Sample qualities were assessed by the total counts of reads (minimum 3 million reads). The RNA-seq results were validated by qPCR in a different group of 28 patients with LRBC who developed CIF after their first cycle of chemotherapy and in six patients who also received chemotherapy but did not develop CIF (controls). Five hundred nanograms of total RNA from these 34 patients were converted into cDNA with the SuperScript® IV First-Strand Synthesis System for RT-PCR (Invitrogen). Amplification reactions were performed in an Applied Biosystems Thermocycler 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, EUA) at a final volume of 15 μL containing: 1X SYBR Green mix (Quantitec SYBR Green PCR kit, QIAGEN cat. no. 204054), 25 pmol of each specific primer designed with the software Primer3 Input 0.4.0) and 2 μL of each cDNA (at an initial dilution of × 10). The thermocycler was programmed for a hot start at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 25 s. We normalized all the RT-qPCR values obtained by the average of the expression of three reference genes (HPRT, β-actin e RPL13a). All primer sequences and their amplicons can be found in Supplementary material.

The gene expression results were obtained using the formula 2^-(ΔCt), and the fold change was calculated by the formula 2^-(ΔΔCt). We normalized all the RT-qPCR values

obtained by the average of the expression of three reference genes (HPRT, β-actin e RPL13a).

Statistical methods

Bioinformatic analysis to identify differences in expression between three patients' PBMNC transcriptome samples obtained before and after chemotherapy was conducted with edgeR software (edgeR: differential expression analysis of digital gene expression data). Read quality control was performed by Thermo Fisher's native primer, trimming, and filtering in Torrent Suite Software. Reads were aligned against a reference of canonical transcripts (hg19_AmpliSeq_Transcriptome_21K_v1) provided by Thermo Fisher Scientific. Counts were obtained by each unique aligned read in gene level. AmpliSeq RNA kit has primers for ~ 21 k genes. Gene filter was based on a minimum count of two reads. If a gene had < 2 counts, it was filtered out of analysis. The method of normalization performed was "TMM" or Trimmed Mean of *M* values, where the weights are from the delta method on binomial data. Dispersion was calculated using estimateDisp function in its defaults values (which uses locfit for dispersion estimation). "Exact test" was used for differential expression. Gene count tables were built by AmpliSeqRNA Plugin default parameters and alignment length < 17 nt were discarded. We chose for the transcriptome analysis patients whose changes in BFI scores were the most striking and who had enough and adequate quality samples for this type of study. Since the distribution of our data was mainly non-parametric, the significance of differences in the expression of each of the selected genes comparing the RT-qPCR results before and after chemotherapy for the 28 patients and six controls was evaluated by the non-parametric Wilcoxon matched pair signed rank test using the Prism-Graph Pad software (https://www.graphpad.com/scientific-software/prism/). We considered statistically significant p values ≤ 0.01 for the choice of the 71 genes (see below): whereas, we considered significant p values < 0.05 for the paired Wilcoxon matched pair signed rank test.

Results

The clinical characteristics of the patients studied here were previously reported [10]. In brief, the mean age of LRBC patients was 48.9 years; 15.6% had stage I, 46.9% stage II, and 37.5% stage III cancer; 68% of patients expressed hormonal receptors, 30% were HER2+ and 28% were triple negative; and 56% received adjuvant therapy, whereas 44% received neoadjuvant therapy. The patients' chemotherapy regimens included doxorubicin, cyclophosphamide (AC) followed by paclitaxel, and paclitaxel associated with trastuzumab (if patients overexpressed Her2).

A total of 12.345 transcripts were sequenced, but only 71 exhibited statistically significant differential expression between samples collected before and after the first cycle of chemotherapy ($p < 0.01$) (Table 1) with a log fold change of more than 5 or less than − 5. Of these 71 genes, 26 were further self-selected based on their important roles in several biological processes potentially involved in CRF, lower p-values and higher absolute fold change in expression. The selected genes were MRPS11, LPIN1, HLTF, TAF1A, ZNF3, DUSP14, DUSP18, and TAF9B (role in gene expression); HIP1R, PAICS, and NAA30 (cellular transport); SFT2D2, GOLM1, SLC37A4, ACACA, KLHL3, RHOBTB1, BST1, PIK3R4, and MS4A6A (cellular metabolism, muscle physiology and enzymatic activity); CHRNG, NCAN, and NBPF14 (ion transport); CGB8 and CCL25 (hormonal regulation); and RAET1G (immune function).

Table 2 shows the means of gene expression of the 26 selected genes before and after chemotherapy for the 28 patients and six controls (patients without fatigue) as well as the significance of the differences between these means. We noted by a significant increase in the expression of DUSP18 and RHOBTB1 and a decrease in the expression of NCAN and RAET1G in patients who developed CIF after Chemotherapy. In the control patients who did not develop CIF, only a significant decrease in NCAN expression could be seen (Fig. 1).

Discussion

In this study, we evaluated changes in gene expression in the PBMNC of LRBC patients who developed fatigue after their first cycle of chemotherapy. Blood lymphocytes have been used as source of RNA in several fatigue-related gene expression studies [13]. Initially, we comprehensively evaluated the transcriptomes of three patients who developed CIF before and after chemotherapy to select genes with significant changes in expression between these two time points. Next, we chose 26 genes to further validate these changes in expression in another 28 patients who also exhibited increases in their fatigue scores after chemotherapy. We also evaluated six controls who did not have increased fatigue scores after chemotherapy. Interestingly, out of the 26 genes selected, only four showed statistically significant changes in expression in the validation step. When we analyzed the physiological roles played by these four genes and found that two of them (RHOBT1 and DUSP18) [14] have important functions in muscle physiology, RAETG1 has an important role in the immune function of NK cells [15] and NCAN may have a role in CNS function [13].

The role of immune system, inflammation, and CNS function in fatigue development has already been described and discussed by others [13, 16–18]. In fact, dysregulation of pro-inflammatory cytokines (mainly IL6, IL1β, and tumor

Table 1 Description of the 71 genes with significantly different expression before versus after chemotherapy. Negative logFC values indicate inverse correlations whereas positive ones imply direct correlations between gene expression before and after Chemotherapy

Category	Gene	logFC*	*p* value	Gene ontology
Ion regulation	CLCNKA	− 9.9451	0.001981	Ion channel activity and voltage-gated chloride channel activity
	CHRNG	− 5.29646	0.007606	Extracellular ligand–gated ion channel activity and channel activity
	ATP2A1	− 8.97845	0.007004	Calcium ion binding and nucleotide binding
	SYT3	− 8.68362	0.005758	Calcium ion binding and syntaxin binding
	NCAN	− 8.10165	0.007514	Calcium ion binding and extracellular matrix structural constituent
	MCTP2	5.939434	0.008018	Calcium ion binding and calcium-dependent phospholipid binding
	REPS1	7.678246	0.007076	Calcium ion binding and SH3 domain binding
	NBPF14	9.570769	0.009586	Calcium ion binding
Gene expression	PCSK9	− 9.08946	0.006828	Poly(A) RNA binding and protein self-association
	PAX4	− 8.31002	0.005453	Sequence-specific DNA binding and RNA polymerase II distal enhancer sequence-specific DNA binding
	MYOD1	− 7.17608	0.009277	Transcription factor activity, sequence-specific DNA binding, and protein heterodimerization activity
	SSX4B	− 6.93653	0.00977	Nucleic acid binding
	MRPS11	5.703083	0.008602	Poly(A) RNA binding and structural constituent of ribosomes
	LPIN1	5.87251	0.007985	Transcription coactivator activity and RNA polymerase II transcription factor binding
	HLTF	5.967245	0.008295	Nucleic acid binding and ligase activity
	HELB	6.016652	0.008716	Poly(A) RNA binding and ATP-dependent DNA helicase activity
	TAF1A	6.108824	0.007937	PEDF-induced signaling and GPCR pathway
	DNAJB4	6.17789	0.006265	Tumor suppressor; unfolded protein binding, and chaperone binding
	DHX32	6.198316	0.007428	Poly(A) RNA binding and ATP-dependent RNA helicase activity
	DUSP18	6.52891	0.005357	Phosphatase activity and protein tyrosine/serine/threonine phosphatase activity
	BRIP1	7.238658	0.005439	Nucleic acid binding and 4 iron, 4 sulfur cluster binding
	ZNF3	7.538883	0.006387	Nucleic acid binding and identical protein binding
	DZIP3	7.88987	0.006955	Poly(A) RNA binding and ligase activity
	ZNF215	8.022671	0.007282	Nucleic acid binding and RNA polymerase II transcription factor activity, sequence-specific DNA binding
	GATA2	8.09043	0.004797	Transcription factor activity, sequence-specific DNA binding, and chromatin binding
	SCML2	8.231816	0.008426	Transcription factor activity, sequence-specific DNA binding
	DUSP14	8.524911	0.002896	Poly(A) RNA binding and protein tyrosine phosphatase activity
	EIF3A	8.593028	0.008445	Poly(A) RNA binding and translation initiation factor activity
	EIF2C3	8.764287	0.009448	Nucleic acid binding and RNA binding
	SIRT7	8.846437	0.009573	Chromatin binding and NAD-dependent histone deacetylase activity (H3-K18 specific)
	KLF9	9.43491	0.005847	Transcription factor activity, sequence-specific DNA binding
	TAF9B	9.209568	0.006105	Protein heterodimerization activity and transcription corepressor activity
Hormones	CGB8	− 8.48591	0.005817	Hormone activity
	CGB7	− 8.31575	0.005856	Hormone activity
	CGB5	− 8.10944	0.00848	Hormone activity
	CCL25	− 7.21392	0.008003	Hormone activity and chemokine receptor binding
Metabolism/enzymatic activity	SH2D4A	− 8.44581	0.008571	Phosphatase binding
	SLC37A4	− 7.93542	0.010827	Transporter activity and glucose-6-phosphate transmembrane transporter activity
	FADS6	− 7.77127	0.009155	Oxidoreductase activity
	EPHA10	− 7.69107	0.006841	Transferase activity, transferring phosphorus-containing groups, and protein tyrosine kinase activity
	TTLL13	− 7.26717	0.009064	Ligase activity
	KLRG2	− 7.23547	0.009869	Carbohydrate binding
	CA9	− 6.58398	0.009858	Carbonate dehydratase activity
	KLHL3	5.47842	0.004154	Actin binding and structural molecule
	HIP1R	5.659792	0.009933	Actin binding and phosphatidylinositol binding

Table 1 (continued)

Category	Gene	logFC*	*p* value	Gene ontology
	NUDT15	6.035628	0.009699	Hydrolase activity and 8-oxo-7,8-dihydrodeoxyguanosine triphosphate pyrophosphatase activity
	ACACA	6.658434	0.006471	Metabolism of water-soluble vitamins and cofactors and initiation of fatty acid biosynthesis
	PAICS	6.829527	0.00795	Purine metabolism
	TTLL5	7.221715	0.009208	Ligase activity
	SDHA	7.249385	0.008142	Oxidoreductase activity, acting on the CH-CH group of donors
	PRPF19	7.394644	0.007381	Identical protein binding and ubiquitin-protein transferase activity
	NUAK2	7.469407	0.008942	Transferase activity, transferring phosphorus-containing groups, and protein tyrosine kinase activity
	RHOBTB1	7.473347	0.009752	GTP binding
	BST1	7.541989	0.007499	Transferase activity and NAD + nucleosidase activity
	DCLRE1A	7.76883	0.007579	5-3 exodeoxyribonuclease activity
	LONRF1	7.79397	0.007306	ATP-dependent peptidase activity
	PDSS2	7.981768	0.008841	protein heterodimerization activity and trans-Octaprenyltranstransferase activity
	STK11IP	8.149778	0.00723	Protein kinase binding
	PIK3R4	9.130203	0.00294	Transferase activity, transferring phosphorus-containing groups, and binding
Cellular transport	STX12	4.950771	0.00736	SNARE binding and SNAP receptor activity (SNARE acts to regulate protein transport between late endosomes and the trans-Golgi network)
	NAA30	6.158729	0.006391	Vesicle-mediated transport and retrograde transport at the trans-Golgi-network
	SFT2D2	7.713047	0.00507	Vesicle transport
	GOLM1	7.761058	0.004921	Not described
Immune function	RAET1G	− 8.01925	0.006675	Peptide antigen binding and natural killer cell lectin–like receptor binding
Others	MAGEA10	− 7.51728	0.007934	Embryonal development and tumor transformation or aspects of tumor progression
	FLJ45079	− 6.79539	0.00911	Not described
	FAM190A	6.576095	0.007258	Serine-rich coiled-coil domain–containing protein 1
	MS4A6A	8.062217	0.008157	Signal transduction as a component of a multimeric receptor complex
	SKIL	8.171628	0.008348	Regulatory role in cell division or differentiation in response to extracellular signals
	FAM72D	9.201304	0.006789	Not described
	SERPINB8	9.663921	0.008906	Epithelial desmosome–mediated cell-cell adhesion

*Log of the fold change (logFC)

necrosis factor: TNF-α) due to single-nucleotide polymorphism (SNP) on its promotor region [17, 18] as well as alterations on its expression [13] has been associated with CRF. But hypothalamic-pituitary-adrenal axis and 5_HT levels dysfunction as well as altered ATP metabolism and muscle metabolism, circadian rhythm disruption and vagal afferent nerve activation [13, 16], oxidative stress, ion transport, or channel activity [17] are mechanisms of CRF pathophysiology also under consideration.

Brown reported that lung cancer patients exhibited significantly worse muscle strength as judged by their slower chair-rising time compared with age-matched controls, and this finding correlated directly with fatigue intensity [19]. Wright reported similar results in survivors of chemotherapy for ALL [20]. Furthermore, Gilliam et al. [4] described the effects of chemotherapy regimens containing doxorubicin (which was also used in our patients) [21], indicating that the increase in the formation of oxidative species could in turn worsen muscle function. The increase in free radicals could lead to the carbonylation of muscle filament proteins, such as actin and myosin [21] contributing to the muscular dysfunction induced by oxidative stress.

RHOBTB1 encodes a protein that belongs to the Rho family of the small GTPase superfamily. This protein plays a role in small GTPase-mediated signal transduction and the organization of the actin filament system [14]. Therefore, under the increased oxidative stress mediated by chemotherapy, skeletal muscle fibers may upregulate RHOBTB1 expression.

A growing body of evidence suggests that the activation of mitogen-activated protein kinase (MAPK) signal transduction mediates changes in muscle gene expression in response to exercise [22, 23]. DUSP18 is a dual-specificity phosphatase

Table 2 Median and standard deviation (SD) values of the BFI and Chalder scores as well as of the 26 selected genes before and after chemotherapy for the 28 patients and 6 controls. *p* values of the non-parametric Wilcoxon matched pair signed rank tests comparing values before and after chemotherapy treatment are also shown

Variable	Patients without fatigue (control)			Patients with fatigue		
	Diagnosis Median (± SD)	Post-chemotherapy Median (± SD)	*p*	Diagnosis Median (± SD)	Post-chemotherapy Median (± SD)	*p*
BFI	1 (± 5.20)	1 (± 5.20)	0.6952	18 (± 17.8)	41 (± 22.8)	< 0.0001
CHALDER	9.5 (± 5.34)	9.5 (± 5.34)	0.1875	10 (± 7.49)	17 (± 5.52)	< 0.0001
MRSP11	0.0249 (± 0.03)	0.0246 (± 0.02)	0.6875	0.0009 (± 0.02)	0.0036 (± 0.50)	0.3965
LPIN1	0.0012 (± 0.003)	0.0022 (± 0.01)	> 0.999	0.0385 (± 0.08)	0.0242 (± 4.31)	0.6226
HLTF	0.0015 (± 0.10)	0.0034 (± 5.55)	0.4375	0.0016 (± 0.01)	0.0014 (± 0.02)	0.6226
TAF1A	0.1016 (± 0.14)	0.0066 (± 0.01)		0.0051 (± 0.06)	0.0357 (± 2.71)	0.1099
ZNF3	0.0121 (± 0.09)	0.1114 (± 0.09)	0.4375	0.0221 (± 0.79)	0.0282 (± 6.81)	0.6848
DUSP14	0.1419 (± 0.05)	0.1660 (± 0.08)	0.6875	0.1340 (± 0.55)	0.3212 (± 11.9)	0.0577
DUSP18	0.1419 (± 0.10)	0.1171 (± 0.03)	0.6875	0.0670 (± 1.25)	0.2590 (± 11.5)	
TAF9B	0.0718 (± 0.05)	0.0570 (± 0.17)		0.0779 (± 0.23)	0.1247 (± 0.31)	0.1815
HIP1R	0.0041 (± 0.01)	0.0074 (± 0.02)	0.1429	0.0359 (± 5.62)	0.0343 (± 1.27)	0.1688
PAICS	0.2569 (± 1.08)	0.3065 (± 0.20)	0.8438	0.1593 (± 18. 9)	0.5994 (± 19.3)	0.0663
NAA30	0.4353(± 0.55)	0.2602 (± 0.17)	0.3290	0.1250 (± 1.37)	0.1710 (± 2.12)	0.1187
SFT2D2	0.0078 (± 0.01)	0.0075 (± 0.01)	0.8438	0.0394 (± 0.04)	0.0335 (± 0.32)	0.7646
GOLM1	0.0458 (± 0.05)	0.0164 (± 0.08)	> 0.999	0.0718 (± 0.09)	0.0452 (± 3.10)	0.3778
SLC37A4	0.0248 (± 1.78)	0.0267 (± 0.03)	0.8125	0.0947 (± 3.88)	0.0211 (± 2.47)	0.2579
ACACA	–	–	–	0.0005 (± 0.26)	0.0007 (± 0.06)	0.1294
KLHL3	–	–	–	0.1211 (± 0.12)	0.0305 (± 21.8)	0.3702
RHOBTB1	0.0048 (± 0.01)	0.0101 (± 0.01)	0.6250	0.0084 (± 0.05)	0.0226 (± 0.05)	0.0079
BST1	0.0156 (± 0.09)	0.0123 (± 0.10)	0.6250	0.0226 (± 0.16)	0.0611 (± 0.47)	0.1447
PIK3R4	0.0230 (± 0.09)	0.0160 (± 0.24)	> 0.999	0.0188 (± 0.27)	0.0611 (± 0.18)	0.2788
MS4A6A	0.0563 (± 0.80)	0.0255 (± 0.30)	0.8438	0.0242 (± 1.70)	0.0432 (± 0.57)	> 0.999
CHRNG	0.0038 (± 0.007)	0.0030 (± 0.01)	> 0.999	0.0103 (± 1.70)	0.0066 (± 0.57)	0.0874
NCAN	0.0125 (± 0.04)	0.0028 (± 0.02)	0.0313	0.0180 (± 0.25)	0.0052 (± 0.06)	< 0.0001
NBPF14	0.1130 (± 5.26)	0.0572 (± 6.77)	0.5625	2.9622 (± 37.0)	1.7411 (± 17.6)	0.4307
CGB8	0.4038 (± 1.50)	0.4329 (± 2.10)	0.2188	3.0366 (± 6.45)	0.6170 (± 11.7)	0.2078
CCL25	0.0231 (± 0.02)	0.0199 (± 0.02)	0.6250	0.0266 (± 0.07)	0.0145 (± 0.05)	0.3755
RAET1G	0.0916 (± 0.16)	0.0460 (± 0.16)	0.4375	0.0720 (± 0.18)	0.0145 (± 0.42)	0.0245

(–) Absence of amplification; *p* values for TAF1A, DUSP18, and TAF9B were not calculable by the software because of too small number of matched samples were available

[24] expressed in muscle [25] that can dephosphorylate both tyrosine and serine/threonine residues, major modulators of critical signaling pathways including MAPK [26]. The significant increase in DUSP18 levels that we observed in patients with CIF may thus decrease MAPK pathway signaling and thus contribute to muscle dysfunction. Furthermore, we also observed an increasing trend in DUSP14 ($p = 0.057$), which helps to corroborate a role for these proteins in CIF. No previous studies have described the association of RHOBTB1 and DUSP proteins with CRF.

RAET1G encodes a member of the major histocompatibility complex (MHC) class I family of proteins. The encoded protein is one of several related ligands of the natural killer group 2, member D (NKG2D) receptor. NKG2D is an activating receptor expressed on the surface of natural killer (NK) cells, $CD8^+$ T cells, subsets of $CD4^+$ T cells, invariant NKT cells (iNKT), and γδ T cells [15]. We cannot explain the relationship between lower levels of RAET1G and fatigue. However, it is possible that lower levels of RAET1G may be a secondary phenomenon induced by high levels of cytokines that may be present in patients with CIF [6, 10, 27].

Interestingly, unlike DUSP18, the RHOBT1B and RAET1G, NCAN levels significantly decreased following chemotherapy in both patients and controls, suggesting that

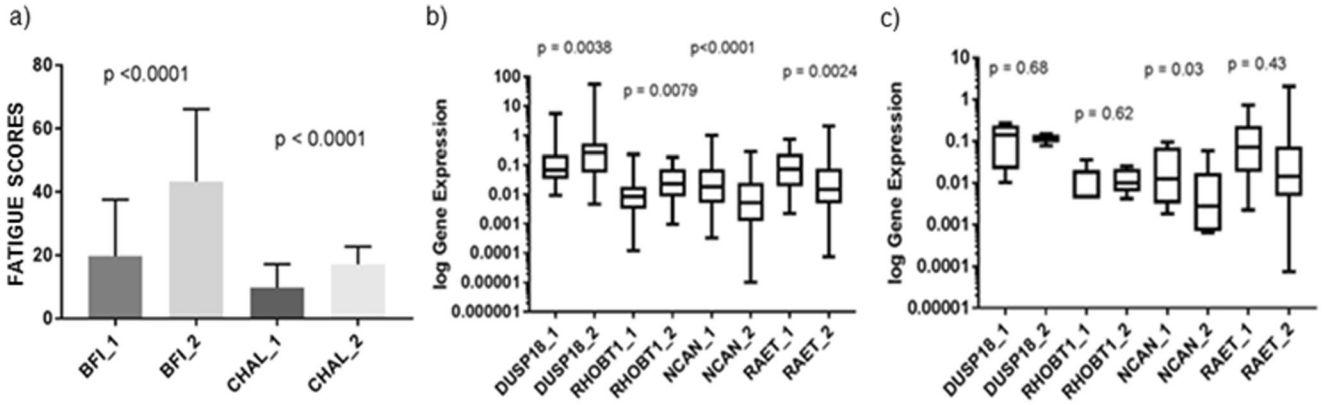

Fig. 1 **a** Patients fatigue score: box plots of the BFI and Chalder Fatigue scores before (BFI_1, Chalder_1) and after chemotherapy (BFI_2, Chalder_2) for the 28 patients who had increased fatigue scores after treatment. **b** Gene expression before and after chemotherapy: box plots of the log-gene expression of DUSP18, RHOBT1, NCAN, and RAETG1 before (_1) and after (_2) chemotherapy for the 28 patients whose fatigue scores increased after treatment. **c** Gene expression data from controls: box plots of the log-gene expression of DUSP18, RHOBT1, NCAN, and RAETG1 before (_1) and after (_2) chemotherapy for the 6 patients (controls) whose fatigue scores did not increase after treatment. *p* values for the comparisons of gene expression before and after chemotherapy appear above each group of two box plots

NCAN expression may decrease as a non-specific effect of chemotherapy. NCAN is a chondroitin sulfate proteoglycan, that is, thought to be involved in the modulation of cell adhesion and migration that may modulate neuronal adhesion and neuronal growth during development by binding to neural cell adhesion molecules (NG-CAM and N-CAM) [22]. In fact, specific NCAN polymorphisms were detected at higher frequencies in patients with dyslexia, schizophrenia and cognitive dysfunction, and at lower levels in patients with mania [28–30]. It is possible that the decrease in NCAN levels that we found in both patients and controls may underlie certain cognitive changes seen in patients undergoing chemotherapy known as chemobrain [31].

Alterations in gene expression associated with cancer-related fatigue have been studied by many other authors [32–37], and all of them highlighted the role of inflammatory and immune system in the development of fatigue. Flowers et al. [32], for instance, using microarray approach, identified alterations in gene expression as well as in biological pathways related to evening fatigue. In their analysis, however, authors used very heterogeneous samples and did not validate their analysis using samples obtained before and after chemotherapy, nor a control group who received chemotherapy but did not develop fatigue. In spite of these differences, inflammation and immune function genes' expression were described as altered in fatigued patients [32]. Bower et al. [33] studied gene expression changes related to persistent fatigue in breast cancer patients. Also, using microarray methodology, these authors focused on inflammation-related genes, especially in the pro-inflammatory NF-kb pathway. They noticed an increased expression of pro-inflammatory transcription factors as well as a decrease expression of anti-inflammatory ones, corroborating the role of inflammatory genes in the development of fatigue. Landmark-Høyvik et al. [34] also described alterations in immune function and in inflammatory system related to fatigue. According to these authors, there was a dysregulation in plasma- and B cell pathways in chronic fatigued breast cancer patients. Those patients, however, were treated with adjuvant radiotherapy instead of chemotherapy.

Thus, to the best of our knowledge, the involvement of RHOBTB1, RAET1G, NCAN, or DUSP18 in CRF or CIF was not previously reported in the literature. Further studies should be conducted to validate our results in other patients with CIF to confirm our data and evaluate whether any of these genes may serve as biomarkers of CIF.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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