Brain, Behavior, and Immunity 27 (2013) 63–70

Contents lists available at SciVerse ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Upregulation of α-synuclein during localized radiation therapy signals the association of cancer-related fatigue with the activation of inflammatory and neuroprotective pathways

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ARTICLE INFO

Article history:
Received 17 May 2012
Received in revised form 14 September 2012
Accepted 18 September 2012
Available online 26 September 2012

Keywords:
Fatigue
Cancer-related fatigue
Genomic
Gene expression
Neuroinflammation

ABSTRACT

Purpose: Neuroinflammatory mechanisms are associated with fatigue in neurodegenerative conditions such as Parkinson's. The symptoms in Parkinson's including fatigue are thought to be related to α-synuclein overexpression. This study investigated genomic correlates of fatigue experienced by men with prostate cancer receiving external beam radiation therapy (EBRT).

Patients and methods: Sixteen men with non-metastatic prostate cancer who were scheduled to receive EBRT were enrolled. Fatigue scores and blood were obtained at baseline (prior to EBRT, D0); one hour following initiation of EBRT (D1), day 7 (D7), day 14 (D14), midpoint (days 19–21, D21), completion (days 38–42, D42), and four weeks post-EBRT (days 68–72, D72). Gene expression profiling using microarray analysis was performed from peripheral blood and confirmatory qPCR and protein (ELISA) analyses verified the microarray results. Correlations between fatigue and gene/protein expressions were determined using a mixed model approach.

Results: Microarray data showed significant, differential expression of 463 probesets following EBRT. *SNCA* had a 2.95-fold change at D21from baseline. *SNCA* expression was confirmed by qPCR ($p < 0.001$) and ELISA ($p < 0.001$) over time during EBRT. Fatigue scores were significantly correlated with *SNCA* gene expression on D14 ($r = 0.55$, $p < 0.05$) and plasma α-synuclein concentrations on D42 of EBRT ($r = 0.54$, $p = 0.04$).

Conclusion: Fatigue experienced during EBRT may be mediated by α-synuclein overexpression. Alpha-synuclein may serve as a useful biomarker to understand the mechanisms and pathways related to the development of fatigue in this population.

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1. Introduction

Localized radiation therapy is one of the main therapeutic options recommended for the management of non-metastatic prostate cancer (Thompson et al., 2007). Advances in prostate cancer treatment using improved techniques in the delivery of localized therapy such as external beam radiation therapy (EBRT) have led to high cure rates and prolonged the natural history of the disease. However, the improved survival rates are mitigated by the toxicities associated with these treatments that lower the quality of life for survivors. Fatigue is one of the most commonly reported and the most distressing side effect reported in the radiation therapy setting, affecting approximately 78% (range = 31–100%) of patients receiving localized radiation therapy (Greenberg et al., 1992; Smets et al., 1998). It is also the most common baseline symptom noted in men with prostate cancer referred for a curative course of radiation therapy (Danjoux et al., 2007) and one of the major indicators of cancer therapeutic outcomes (Monga et al., 1999). Fatigue severity of most men with prostate cancer is known to increase significantly during the course of radiation therapy peaking at midpoint and declining after completion of therapy (Miaskowski et al., 2008). The etiology of fatigue progression and severity while receiving cancer treatment is currently unknown. However, neuroinflammation has been reported to be related to fatigue in other conditions (Bokemeyer et al., 2011; Rönnbäck and Hansson, 2004).

Although the National Comprehensive Cancer Network (NCCN) practice guidelines recommend the use of methylphenidate as a

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0889-1591/$ - see front matter Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.bbi.2012.09.009

pharmacological intervention for cancer-related fatigue (CRF) (Mock et al., 2000) based on evidence from small, single arm trials (Bruera et al., 2003; Hanna et al., 2006), randomized clinical studies failed to validate these results (Bruera et al., 2006; Butler et al., 2007; Mar Fan et al., 2008). There is currently no optimal pharmacologic therapy and scant molecular evidence to guide the development of effective therapies for the management of cancer- and/or cancer treatment-related fatigue. This hypothesis-generating study explored genomic correlates of cancer-related fatigue by investigating changes in gene expression specifically focusing on those genes that are associated with neuroinflammation and their relationship with fatigue scores during EBRT. This approach may assist in understanding the contribution of neuroinflammation in the development of cancer-related fatigue and may provide new insights for potential interventional targets. This study uses an unbiased approach to demonstrate the associations between differential gene expression changes from peripheral blood and fatigue scores over time during EBRT.

2. Methods

Men with non-metastatic prostate cancer were enrolled under an actively recruiting protocol 09-NR-0088 (NCT00852111). Data collection was conducted from May 2009 to December 2010. Study outcomes were measured at baseline (prior to EBRT, D0); one hour following initiation of EBRT (D1), day 7 (D7), day 14 (D14), at midpoint (days 19–21, D21), at completion (days 38–42, D42), and four weeks post-EBRT (days 68–72, D72). Patients with progressive disease causing significant fatigue; psychiatric disorder within five years; uncorrected hypothyroidism or anemia; taking sedatives, steroids, or non-steroidal anti-inflammatory agents; or second malignancies were excluded. Fatigue was measured at each time point using the revised Piper Fatigue Scale (rPFS), a 22-item paper/pencil questionnaire that has a zero to ten rating scale (0 = none; 10 = worst intensity) and defines severe fatigue as a score of >6 (Piper et al., 1998). Only subjects with rPFS scores and corresponding quantitative real-time polymerase chain reaction (qPCR) data were included in the final analysis. Depressive symptoms were also assessed at each time point using the clinician-administered, 21-item Hamilton Depression Rating Scale (HAM-D), which has been validated in other fatigue studies (Lydiatt et al., 2008).

Blood for gene expression, responses to fatigue and depression questionnaires were also obtained during one outpatient visit from age, gender, and race-matched healthy controls enrolled under protocol 09-NR-0131 (NCT00888563). Data from these healthy volunteers were used to compare baseline (D0) data obtained from study subjects in order to establish that study subjects are similar to healthy individuals prior to EBRT. Exclusion criteria for healthy controls were: confirmed medical condition causing clinically significant fatigue; taking medications known to cause fatigue; worked late evening and night shifts within the past month; reported a severe psychiatric condition; consuming >300 mg of caffeine-containing beverages or >1 lb of chocolate a day; consuming >2 servings of alcohol-containing beverages everyday; and having detectable blood alcohol content. Both protocols were approved by the Institutional Review Board of the National Institutes of Health (NIH), Bethesda, Maryland, USA.

2.1. Gene expression chip processing and pathway analysis

For an unbiased, hypothesis-generating approach, several steps were chronologically followed in the study. First, an initial gene expression profile was explored using microrarray technology to determine a list of differentially expressed genes during EBRT. At each time point, 2.5 mL of blood from subjects was collected using RNA PAXGene tubes (Qiagen, Frederick, Maryland) for each of the seven time points. The collected blood was stored at −80 °C in a freezer until ready for RNA extraction. RNA extraction, purification, cDNA and cRNA synthesis, amplication, hybridization, scanning and data analyses were conducted following standard protocols as previously described (Wang et al., 2007). A total of 80 Affymetrix microarray chips (HG U133 Plus 2.0, Santa Clara, California) were used for gene expression analysis. Affymetrix GeneChip Command Console (AGCC, 3.0 V) was used to scan the images for data acquisition. Raw signal intensity values were normalized using S10 transformation algorithm from the MSCL Analyst's Toolbox. S10 transformation is a variance stabilizing, quantile normalization transform and is scaled to match a base 10 logarithm. S10 values were subjected to principal component analysis in order to detect outliers. Seven chips were identified as outliers and excluded from further analysis. The remaining transformed data were subjected to linear regression analysis adjusted for patient effect with respect to the seven time points treated as equal intervals. The slope measured the trend of expression change between baseline through D72.

Ingenuity Pathway analysis (Ingenuity® Systems, www.ingenuity.com, Redwood City, California) identified functional networks of the differentially expressed probesets from the Ingenuity's Knowledge Base. Right-tailed Fisher's exact test was used to calculate *p*-values determining the probability that each biological function and/or disease assigned to these networks was due to chance alone.

2.2. Confirmatory quantitative real time polymerase chain reaction (qPCR)

Second, in order to confirm the differentially expressed genes in the microarray experiment, qPCR was performed from the same RNAs used in the microarray experiment in all seven time points for the study subjects and at one study time point for the matched controls. Total RNA was isolated with an RNA kit and treated with DNase I during purification. First strand cDNAs were synthesized using RT^2 First Strand Kit (Qiagen, Frederick, Maryland) with 100 ng of total RNA from each sample and subsequently diluted tenfold with dH_2O. The qPCR amplification mixers (10 μL) contained 1 μL of diluted first strand cDNA, 5 μL of 2× RT^2 Real Time SYBR Green/Rox PCR Master Mix (Qiagen, Frederick, Maryland) and 400 nM of forward and reverse primers. Reactions were carried on ABI PRISM 7900HT Sequence Detection System and were subjected to initial ten minute denaturation at 95 °C and 40 cycles at 95 °C for 15 s and 60 °C for 60 s.

Five potential reference genes were tested including *B2M* (beta-2-microglobulin), *HPRT1* (hypoxanthine phosphoribosyltransferase 1), *RPL13A* (ribosomal protein L 13a), *GAPDH* (glyceraldehydes-3-phosphate dehydrogenase) and *ACTB* (actin,beta). *GAPDH* and *ACTB* were validated and chosen as reference genes. Primers for *GAPDH* (reference position 1287), *ACTB* (reference position 1222) and *SNCA* (reference position 876) (Qiagen, Frederick, Maryland) had efficiencies between 90% and 110%. When calculating for ΔCt values, geometric means of Ct values of the 2 reference genes were used.

2.3. Confirmation by ELISA

Third, protein levels from plasma separated from whole blood collected from the same patients in the microarray experiment using EDTA tubes in all seven time points and stored at −80 °C were quantified using human protein ELISA kits (Invitrogen, Camarillo, California). ELISA was performed using 50 μL of plasma samples according to manufacturer's guide. The plates were read in a microplate reader VICTOR3 at 450 nm. All samples were tested in triplicate.