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14. ABSTRACT This project is based on the premise of developing a new method to identify and establish the presence of genomic instability mediated chromosomal heterogeneity in Gulf War Illness patients. In our previous research, we successfully established a link between genomic instability (measured by elevated frequencies of non clonal chromosome aberrations (NCCAs)) and cancer progression. During the current study period of this DOD supported grant, we have analyzed blood samples from patients with GWI using multiple-color spectral karyotype profiling (SKY). Thus far, elevated NCCAs frequency has been observed in all GWI patient samples tested so far representing a statistically significant finding. To date we have tested 10 GWI patients and 20 controls. We now believe that our methodology represents a very promising means of establishing a clinical biomarker based on overall genome instability in GWI patients. We further anticipate that the findings of this research project will lead to support for translational projects that will result in vastly improving diagnosis and assessment of GWI patients.					
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Introduction

The objective of this proposal is to determine whether there is a link between genome instability (reflected as elevated frequencies of NCCAs) and GWI. Patient samples (blood) are being directly analyzed using molecular cytogenetic analyses and gene expression studies. *The central hypothesis of this proposal is that patients with GWI will display high levels of genome instability, particularly when challenged by various toxic agents, which can be stochastically linked to various molecular pathways and is the potential basis for diverse clinical symptoms that are seen in GWI.*

Specific Aim 1: Establish a method to determine levels of genome variation/NCCAs in blood cells (lymphocytes) to monitor overall genetic instability in patients with GWI.

Specific Aim 2: Link high levels of genome instability to various gene level alterations or molecular pathways illustrated by gene expression studies and copy number variation analysis.

Purpose and scope of the research effort: These specific aims will systematically link genomic instability as detected in the blood cells of veterans with detectable genetic alterations. It is anticipated that this study will form the basis of new methodology that can be translated to prescreen veterans for the likelihood of developing GWI or used to diagnose GWI in veteran populations. The molecular characterizations proposed in Specific Aim 2 become important if Specific Aim 1 fails. If Specific Aim 1 is successful, attention will be focused on the expression profiles rather than in vitro experiments to induce genome instability. Based on the exciting data/results so far from Specific Aim 1, Specific Aim 2 will be slightly modified (see “Conclusions”-part e.).

Body

To date, our lab has tested 10 GWI patients and 20 controls (both from military and non-military populations). Current data strongly supports our hypothesis that GWI patients display significantly elevated genome instability.

A. Materials and Methods:

1. Chromosome preparations were performed on 10 patients and 20 controls: Among the samples received, we have successfully harvested chromosomes from the short term cultures listed below.
2. SKY experiments have been carried out on all samples. Standard protocols were used. Briefly, following pepsin treatment and fixation with formaldehyde followed by dehydration, the chromosomal slides were denatured in 70% formamide and 2 X SSC and hybridized with denatured mouse or human painting probes for over 48 hours at 37°C. Signals were detected following slide washing. Mitotic figures with good hybridization quality were randomly captured using a CCD camera. Following image acquisition, chromosomes were karyotyped according to their color and size with Applied Spectral Imaging software (Liu et al., 2000; Heng et al., 2001, 2003; Ye et al., 2001).
3. Approximately 100-200 SKY images were randomly captured for each sample. At least 50-200 images of good quality were analyzed in detail. All chromosomal aberrations were scored. The frequencies of chromosomal aberrations (percentage of NCCAs among all examined mitotic figures) were summarized.

B. Results:

1. NCCA frequencies of 10 controls from a military population:

#control 1	4%
#control 2	8%
#control 3	2%

#control 4	1%
#control 5	1%
#control 6	4%
#control 7	0%
#control 8	4.2%
#control 9	0%
#control 10	7.4%

Average frequency: 3.16%

2. The average frequency of NCCAs from a non-military population is slightly lower.

#1	0%
#2	1%
#3	0%
#4	3%
#5	0%
#6	7%
#7	0%
#8	2%
#9	2%
#10	0%

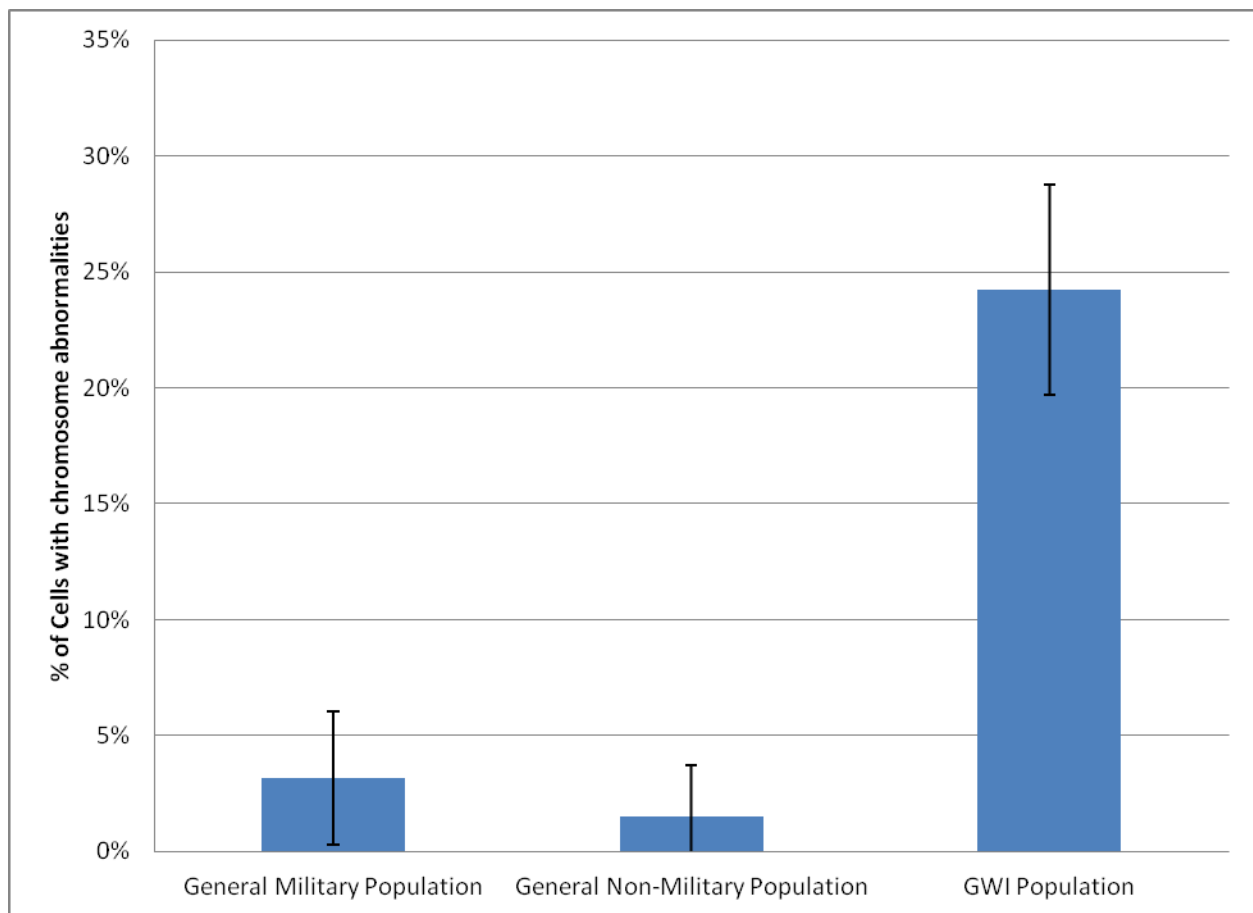
Average frequency: 1.5%

3. The frequencies of NCCAs from GWI Patients:

#11	23%
#12	30%
#10	27%
#7	20%
#B1	18%
#B2	30%
#B3	19%
#B4	26%
#B5	25%
#B6	N/A

Average frequency: 24.2%

4. Data comparison (**Fig 1**):



Chromosome abnormalities are significantly increased in the GWI population (n=10) compared with both the Military (n=10 $p=2.2\text{E-}08$) and Non-Military (n=10 $p=3.0\text{E-}08$) populations.

C. Current additional experiments and future works:

1. Conventional cytogenetic analyses:

Interestingly, if judged by only stochastic translocations without accounting for chromosomal breakages and other types of aberrations, some GWI patients display a lower rate. In our current study, NCCAs include different types of chromosomal aberrations. To compare the contributions of different types of chromosomal aberrations, conventional cytogenetic analyses were also used to provide further characterization. One interesting finding is the elevated rate of defective mitotic figures observed in some patients. DMFs are a new type of chromosomal aberration and reflect the condensation errors. As errors occurring during the condensation stage can also generate chromosomal breakage and translocations, DMFs could be a promising biomarker linked to GWI patients.

Giemsa stained chromosome slides were used to score various chromosomal abnormalities. In our ongoing experiments, 100-200 mitotic figures per patient/control will be studied. The types of aberrations will be used and correlated with a patient's clinical profile. In addition, one of the potential

biomarkers termed C-Frag (a newly identified biomarker for mitotic cell death) has been identified in some of the samples. Further studies are currently ongoing to confirm these observations. Both DMFs and C-Frag are potential biomarkers with clinical relevance that could be used in both diagnosis and possible disease progression assessment.

2. RNA preparation for Specific Aim 2: All patients and control RNA have been prepared. The gene expression profile will be analyzed after all samples are collected. This process will last until near the end of the research project.

Key Research Accomplishments

1. Continued further successful refinements in methodology applications to be used in GWI patient populations have been done over the past year.
2. Recently, more of our manuscripts have been published and the DOD's support has been cited. The following papers have been published during the reporting period:

Stevens JB, Abdallah BY, Liu G, Ye CJ, Horne SD, Wang G, Savasan S, Shekhar M, Krawetz SA, Hüttemann M, Tainsky MA, Wu GS, Xie Y, Zhang K, Heng HH. Diverse system stresses: common mechanisms of chromosome fragmentation. *Cell Death Dis.* 2011 Jun 30;2:e178. doi: 10.1038/cddis.2011.60.

Heng HH, Liu G, Stevens JB, Bremer SW, Ye KJ, Abdallah BY, Horne SD, Ye CJ. Decoding the genome beyond sequencing: The new phase of genomic research. *Genomics.* 2011; 98: 242-52

Heng HH, Stevens JB, Bremer SW, Liu G, Abdallah BY, Ye CJ. Evolutionary mechanisms and diversity in cancer. *Adv Cancer Res.* 2011; 112:217-53.

Heng, H. H., Liu, G., Stevens, J. B., Abdallah, B. Y., Horne, S. D., Ye, K. J., et al. (2013). Karyotype heterogeneity and unclassified chromosomal abnormalities. *Cytogenet and Genome Research*, In press.

Reportable outcomes

1. Preliminary samples that include 10 GWI patients and 20 controls indicate significant correlation with genomic instability identified in GWI patients when compared to controls.
2. Potential biomarkers have been identified that might be present in GWI patients that could be used for diagnosis and possible disease severity/progression prognosis correlation.

Conclusions:

- a. The patient samples analyzed so far display elevated chromosomal aberrations as determined by SKY analyses. This is a very important finding. With the examination of additional patients followed by statistical analyses, we anticipate that we will be able to report this exciting new link between genomic instability and GWI.
- b. Lab experimental protocols are successful and are being further refined for use in GWI patient populations. One example of this is the use of interphase FISH to confirm the involvement of aneuploidy, as the detection of polyploidy has been higher in patients.
- c. We have discovered data differences in control groups. Due to this finding we also will compare more samples between the military and non-military populations.

- d. Due to the identification of potential biomarkers, we are developing additional cytogenetic characterization to systematically link potential sub-groups of specific types of chromosomal aberrations. Methodology applications are also being considered for use in commonly associated GWI comorbidities (e.g. Myalgic Encephalomyelitis/Chronic Fatigue Syndrome).
- e. Regarding Specific Aim 2, based on the results of our initial data, we will not focus on using in vitro induction of instability because genome instability is directly evident in all patients' blood cells tested. Based on the new model that stress triggers the genome to be unstable and is likely the common cause of GWI, expression studies will become an important link connecting diverse patients through common stress pathways. Therefore, the transcriptome profile of all patients will be the new focus of Specific Aim 2.

References

Stevens JB, Abdallah BY, Liu G, Ye CJ, Horne SD, Wang G, Savasan S, Shekhar M, Krawetz SA, Hüttemann M, Tainsky MA, Wu GS, Xie Y, Zhang K, Heng HH. Diverse system stresses: common mechanisms of chromosome fragmentation. *Cell Death Dis.* 2011 Jun 30;2:e178. doi: 10.1038/cddis.2011.60.

Heng HH, Liu G, Stevens JB, Bremer SW, Ye KJ, Abdallah BY, Horne SD, Ye CJ. Decoding the genome beyond sequencing: The new phase of genomic research. *Genomics.* 2011; 98: 242-52

Heng HH, Stevens JB, Bremer SW, Liu G, Abdallah BY, Ye CJ. Evolutionary mechanisms and diversity in cancer. *Adv Cancer Res.* 2011; 112:217-53.

Appendices