

**INVESTIGATING THE ROLE OF VITAMIN D AND DNA REPAIR IN
INFLUENCING CANCER PRESENTATION AND OUTCOMES**

An Undergraduate Research Scholars Thesis

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ABSTRACT

Investigating the Role of Vitamin D and DNA Repair in Influencing Cancer Presentation and Outcomes. (May 2015)

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Recent studies have identified differences in cancer risk, severity, and response to treatments in different ethnic groups. When comparing Americans of African descent to those of Caucasian descent, symptoms in African American patients were consistently severe with increased mortality rates. Research has indicated that this difference in the cancer phenotype between these two ethnic groups may be a result of both biological and socioeconomic factors¹. Our current study will focus on the potential- biological factors. We hypothesize that vitamin D deficiency in

the AA population and associated differences in DNA repair capacity are the biological basis of the cancer- phenotypic variance between these populations.

Lymphoblastic (LCL) cell lines cataloged in (<http://www.1000genomes.org/>) with known genotypes of human repair genes will be quantified for DNA repair capacity using comet assay, cell cycle analysis, and gene expression of key DNA repair genes (for both ethnic groups) after exposure to DNA damaging chemotherapeutic agents. Chi-square based population association approach will be used to associate genotypes of DNA repair genes to DRC capacity, thus providing the basis of population difference in the cancer phenotype.

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NOMENCLATURE

AA	African American
CA	Caucasian American
DRC	DNA Repair Capacity
PCA	Principal Component Analysis

CHAPTER I

INTRODUCTION

Several socioeconomic and biological factors have been shown to influence the disparity in cancer presentation and outcomes in AA and CA. Despite socioeconomic factors, such as late stage clinical presentation and decreased access to health care resources, being shown to have an influence on cancer outcomes, differences in the two populations persisted even after matching them for socioeconomic conditions. Several parameters including clinical, biological and cellular characteristics such as mortality, age of onset, hormonal status, S phase status, and tumor size, differed between the populations¹⁻³. A strong link between DRC and cancer propensity has been well documented in the current literature³. Furthermore, recent research demonstrates that the serum concentration of vitamin D is directly related to the DRC of cells and thus propensity to develop cancer^{4,5}. Vitamin D deficiency in AA population is known and has been concomitant to population movement from tropical zones (more direct sunlight) to temperate zones (less direct sunlight)^{6,7}. The vitamin D deficiency identified in the AA population in the United States thus may be attributed to decreased absorption of Vitamin D that is modulated both by available sunlight and melanin pigments of the skin⁶⁻⁸. Although it is clear that there is a biological component in the cancer disparity between the AA and CA populations, there is no clear explanation as to the underlying cause behind this dissimilarity^{3,8}.

Here, we address this gap in knowledge by proposing a novel, testable hypothesis. We hypothesize that difference in cancer severity between the two populations is due to differences in DNA repair capacity associated with Vitamin D deficiency. To test this hypothesis, we will evaluate the extent of DNA repair after exposure to commonly prescribed chemotherapy agents with and without Vitamin D in AA and CA population. We expect to find differences in DRC linked to DNA repair enzyme polymorphism frequencies in the population and vitamin D concentration in the serum.

Vitamin D and DNA Repair

DNA damage is a relatively common natural phenomenon in which DNA acquires a mutation or is broken. A normal cell will be able to undergo DNA repair in response to the damage by a variety of mechanisms. Repair mechanisms mend damages, which would otherwise have interrupted with DNA's principal functions of replication and transcription, and correct DNA mutations⁹. Cells that are unable to sufficiently repair DNA can begin to accumulate damage, which can lead to genomic instability¹⁰. Furthermore, patients with diminished DNA repair ability have been found to be at much higher risk for breast cancer than patients with normal levels of DNA repair⁵.

Vitamin D is a known cofactor of many biological pathways, including DNA repair, so it is assumed that the level of vitamin D in the body may affect cancer risk and severity. Deficiencies in vitamin D are linked to increased frequencies of DNA breaks, both single and double

stranded, due to elevated levels of stress¹¹. Some of the populational differences in cancer characteristics are attributed to gene variants (SNPs) that may directly affect the vitamin D metabolism pathways, resulting in varied serum concentration levels among different populations¹². Increasing the amount of serum vitamin D could promote repair of damaged DNA, thus helping to maintain DNA integrity and stability. This in turn would lead to a reduction in the levels of damaged DNA present in the cells, which could help decrease breast cancer risk¹³.

Techniques

By challenging Lymphoblastoid cell lines (LCLs) with known genotypes from both AA and CA through exposure to DNA damaging chemotherapeutic drugs, and measuring the amount of DNA repair when supplemented with vitamin D, we plan to correlate the phenotypic observations with genotypes using the various experimental techniques described as follows:

Cell Cycle Analysis

Cell cycle analysis is utilized to determine the amount of cell aggregates in each phase of the cell cycle. This is evaluated by staining cells with Propidium Iodide and analyzed using flow cytometry.

Real-Time PCR

To determine the transcriptomics of DNA repair gene pathways, Real-time PCR is conducted by isolating the cellular RNA and reverse transcribing it to cDNA. Two-hundred genes in six major DNA repair pathways will be analyzed for gene expression, allowing us to determine gene alterations in DNA repair pathways.

Based on the data, we intend to expand our research focus to discovering specific DNA repair pathways that differ between the two populations. By applying the knowledge of the clinical (phenotypic) differences in presentation and outcome between these populations to molecular underpinning behind (genotype) them, we plan to identify factors that can be exploited to eliminate cancer disparities. This study will prove beneficial in the cancer survival outcomes in both AAs and CAs.

CHAPTER II

MATERIALS AND METHODS

Selection of Cell Lines

Twenty-four Lymphoblastoid cell lines (LCLs) were purchased from the Coriell Institute (NJ, USA) and were selected based on high fixation index (F_{st}), which is a measure of population differentiation due to genetic structure in their SNPS that discerned them from each other. To avoid genomic admixture issues, the cell lines were selected from two independently segregating populations: Caucasian (Utah (USA) Families, CEPH) and African (Nigerian families, YRI). Twelve cell lines were obtained from the Caucasian populations and 12 cell lines were obtained from the African populations. The SNPs were chosen from genes participating in DNA repair and Vitamin D metabolism pathways. All of these cell lines had been obtained and anonymized by the National Institute of General Medical Sciences prior to deposit, and all subjects had provided written consent for the use of their DNA and cells for experimental purposes.

Tissue Culture Methodology

LCL cell lines were maintained in RPMI medium 1640 with 1% L-glutamine (GIBCO) supplemented with 10% FBS (Atlanta, Biologicals) in T-25 tissue culture flasks. Cell counts were made and trypan blue exclusion assays were used for cell viability estimation. At the time of experimentation, cell lines were >95 % viable.

Chemotherapy Drugs and Vitamin D Exposure

Cells were seeded at a density of 2×10^5 cells/well in 24-well cluster plates (Sigma) and the following treatments were applied in triplicates for 12 hours: with chemotherapy drugs, with vitamin D, with drugs and vitamin D, and untreated controls. The cells were then transferred to 15 mL tubes, washed with PBS, and seeded back with or without Vitamin D (Calcitriol, Tocris Biosciences) at 10^{-7} M final concentrations for another 12 hours. The cells were then removed, washed with cold PBS, and fixed in 70% ethanol (v/v) overnight at 4° C. For the chemotherapy drugs Cyclophosphamide and Doxorubicin, which were used in combination to mimic typical clinical protocols, the concentrations chosen were 800ug/ml and 80ug/ml respectively. For Cisplatin, the concentration used was 0.15mM.

Cell Cycle Analysis

The next day, the collected cells were centrifuged at $800 \times g$ for 10 minutes. They were then washed in ice cold Phosphate Buffered Saline (PBS) twice and treated with ribonuclease (100 ug/ml) (DNase free, Sigma) in PBS for 30 minutes at room temperature. Finally, the cells were resuspended in 50 mg/L propidium iodide (Sigma) in PBS and were immediately subjected to flow cytometry optimized for propidium iodide using a fluorescence-activated cell sorting (FACS) (BD Accuri™ C6Scan, San Jose, CA). Appropriate settings of forward and side scatter gates were used to examine 10,000 cells/experiment. Results were analyzed with Cell Quest (Becton Dickinson) and Modfit (Verity Software House, Topsham, ME) software.

Statistical Analysis

Statistical analyses were performed using a standard one-way ANOVA with Dunnett's post-test (InStat software, GraphPad Software, San Diego, CA). Differences with $P < 0.05$ were considered significant.

RNA Isolation and Characterization

RNA was isolated from LCLs after appropriate treatments in triplicates using TRIZOL reagent (Invitrogen) as per the recommended protocol. Briefly, 1 ml of reagent was used per well followed by the addition of 200 μ l chloroform. The aqueous phase was separated by centrifugation in a microfuge. RNA was precipitated by adding 250 μ l isopropanol and isolated as a pellet by centrifugation. The pellet was then washed sequentially with 80% and 100% ethanol, and air-dried. RNA was dissolved in DEPC-treated H₂O, and stored in aliquots at -80°C .

The concentration of RNA was determined by absorbance measurements at 260 nm in a nanodrop spectrophotometer. The presence of contaminating proteins and organic compounds, such as phenol, in the RNA preparation were checked by determining absorbance ratios at 260 nm/280 nm and 260 nm/230 nm, respectively. The quality of RNA was characterized by electrophoresis in 1.2% Agarose gel. The electrophoresis allowed the quantitation of 28S rRNA and 18S rRNA. As mammalian 28S and 18S rRNAs are approximately 5 kb and 2 kb in size, a 2:1 ratio is considered the benchmark for intact RNA.

cDNA Synthesis and Gene Expression Quantification using QPCR

10 μ l RNA of 50 ng/ μ l was reverse-transcribed in 40- μ l reactions. Reverse transcription (RT) was performed using the miRCURY LNA Universal RT microRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon). cDNA was diluted 50 \times and assayed in 10 μ l PCR reactions according to the protocol for miRCURY LNA Universal RT microRNA PCR. Each miRNA was assayed in triplicates. The amplification was performed in a CFX Touch Real-Time PCR System (BIO-RAD) in 384-well plates. The amplification curves were analyzed using the CFX ManagerTM software, both for determination of the quantification cycle (C_q; by the second derivative method) and for melting-curve analysis. The amplification efficiency was calculated using algorithms similar to the LinRegPCR software. All assays were inspected for distinct melting curves and the melting temperature was checked to be within known specifications for the assay. Furthermore, assays detected with C_q < 37 were included in the data analysis as per manufacturer's instructions. Data that did not pass these criteria were omitted from any further analysis.

The test miRNA quantification normalized data to the level of miR-425, as miR-425 levels were expressed close to the global mean in the screening cohort and miR-425 previously has been found valid as a housekeeping miRNA.

mir-let7f
mir-let7p-5-p
mir-21
mir-22
mir-26-5p
mir-26b-5p
mir-34a
mir-108
mir-138
mir-146a
mir-152
mir-155
mir-182
mir-185
mir-196a-5p
mir-200c
mir-421
mir-1005p
mir-1256
mir-1296
mir-1925p
mir-3200-3p

Table 1: List of 22 miRNA.

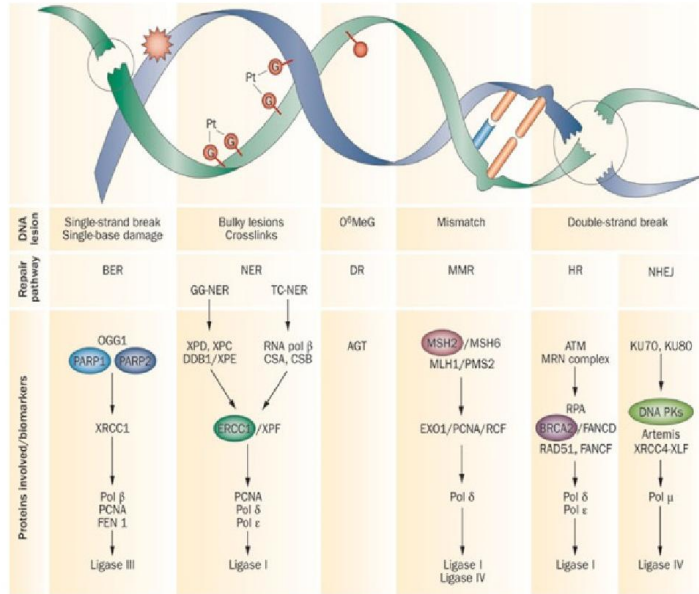
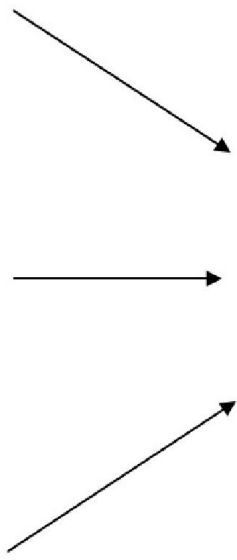


Figure 1: Six DNA repair pathways targeted by the 22 miRNA listed in Table 1. <http://jonlieffmd.com/blog/the-many-ways-neurons-repair-their-own-dna>

Statistical analysis

The results were expressed as mean + SD. Differences between the groups were evaluated by analysis of variance, followed by the Tukey–Kramer test for post hoc analysis. A p-Value less than 0.05 was considered statistically significant.

Signal Pathway analysis of miRNA

Ingenuity pathway analysis (IPA) software (Ingenuity Systems, Redwood City, CA; available at: <http://www.ingenuity.com/>) was applied for Bioinformatic analysis to analyze the canonical pathways, networks, and biological functions of miRNAs. IPA network diagrams were generated based on their score, which is calculated by taking the negative log of the P-value for the likelihood these molecules would be found together by chance alone. A higher score indicates greater statistical significance that molecules depicted in the network are interconnected. Each network includes several partner molecules that were assigned to the network by IPA, some of which are not among the input focus genes. The latest version (17199142, Release Date: 17 September, 2013) was used.

CHAPTER III

RESULTS

Cell Cycle Analysis

We expect to observe statistically significant genetic-association between SNPs' in the DNA repair pathway and the quantity of cells arrested in S-phase based on the ability of the SNPs to modulate DNA repair, when cell lines are treated with chemotherapeutic agents. Addition of vitamin D as when compared to the cell lines treated only with the drugs may show the effect of vitamin D in DNA repair which may in turn be related to the SNP based polymorphism of the vitamin D pathway genes of the corresponding cell lines.

Initial results have supported our expectations that chemotherapeutic agents would lead to an accumulation of cells in the S-phase and that differential clustering occurs based on treatment and genome. A large difference in the F_{st} value of genes in Caucasian American and African cells lines was found. This allelic difference could be influencing DNA repair efficiency and therefore the number of cells in the S-phase.

Coding and Non-coding DNA Repair Transcriptome

To identify specific gene expression and gene networks associated with the 6 common DNA repair pathways and their targeted top 22 miRNA's. We will perform Quantitative real-time PCR. We expect to observe gene expression patterns and thus DNA repair transcriptomic-

networks to differ between African and Caucasian population derived cell lines thus suggesting DNA repair as one significant cause of differences between presentation of tumor biology and cancer outcomes between African and Caucasian patients.

CHAPTER IV

DISCUSSION

Recent studies have found a difference in cancer severity between Caucasian Americans and African Americans. While this difference can be explained in part by socioeconomic conditions, here we focus on the second component, the biological basis of the difference. The 1000-genome project (<http://www.1000genomes.org/>) database provides a tool to identify SNPs in the DNA repair pathways that may contribute to biological differences in cancer presentation and outcome between the two ethnic groups. Several DNA repair genes showed a fixation index (F_{ST}) value greater than 75%. F_{ST} is a measure of population differentiation due to genetic structural differences based on allele frequencies. This difference in the F_{st} values of African and Caucasian American cells suggests that genetic differences in DNA repair genes exist, which may in turn contribute to underlying differences in DNA repair capacity. These genes with high F_{st} value represent all six known common DNA repair pathways and DDR response. This supports our hypothesis that genetic variations exist in the DNA repair genes and that certain alleles may provide advantages in repair capacity. Our results are in agreement with several recent reports suggesting a causal link between genes from DNA repair and cancer presentation¹⁴⁻¹⁶.

In our study, HMGB1 and TP53 are important genes with high F_{st} values. HMGB1 enhances transcription and has recently been found to be a cytokine that plays a role in immunological

responses¹⁷. Previous research has suggested that high levels of HMGB1 expression may support cancer cell proliferation. When HMGB1 expression was down-regulated, prostate cancer cells underwent apoptosis decreasing cancer cell number and growth¹⁸. Because there is a large variation in the gene between the two populations, the level of HMGB1 may be an important gene influencing the disparity in cancer symptoms. TP53, also known as “tumor protein 53,” is a well-known tumor suppressor that acts as a checkpoint regulating cell replication. It binds DNA strands and determines if there is any DNA damage. If damage exists, it activates repair pathways or apoptosis. A mutation, or SNP, in this gene could interfere with its ability to inhibit accumulation of DNA damage, restrict unchecked replication of cells, and disrupt tumor formation. The high percentage differences in the SNPs of this gene may also lead to variations in functional efficiency.

By examining the genomic basis of biological variation, one of the aspects of racial and ethnic disparities in disease patterns, research such as this may shed light on the etiology of many complex diseases and identify new approaches to prevent these diseases in high risk populations. Results obtained from the cell cycle analysis showed increased S-phase arrest in cells treated with chemotherapeutic agents than cells in control groups. This is expected because the chemotherapeutic agents damage the DNA preventing it from passing the replication checkpoints in the S-phase. Genetic association studies of S phase arrest with SNPs in DNA repair pathways will shed light on the influence of specific SNPs on S phase progression and underlying DNA repair capacity. This work is currently in progress.

Based on the data we have obtained thus far, we intend to expand our research focus to discovering specific DNA repair pathway signaling networks that differ between the two populations. By applying the knowledge of the clinical (phenotypic) differences in presentation and outcome between these populations to molecular underpinnings behind them (genotype), we plan to identify factors that can be exploited to eliminate cancer disparities. This study will not only prove beneficial in the cancer survival outcomes in African Americans and Caucasians, but will be a pivotal breakthrough in our quest towards personalized medicine.

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