

**HEALTH BEHAVIORS AND BREAST CANCER RISK IN NON-HISPANIC WHITE & HISPANIC WOMEN**

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**Purpose**

Approximately a third of breast cancer incidence is related to lifestyle choices, and the risk for developing the disease varies among ethnicities [1]. Cigarette smoking, alcohol consumption, body size and shape, physical activity, and diet are all modifiable factors associated with breast cancer risk [1-3]. However, much is known of the combined effects of the risk factors. Some epidemiological studies suggest that a combination of unhealthy risk factors is associated with increased cancer risk [4,6]. There is also little information on minority populations. This study developed a healthy behavior index (HBI) and compared its association with breast cancer risk in non-Hispanic white (NHW) and Hispanic (H) women.

The main objective was to discover if the healthy behavior index is associated with breast cancer risk by case-control status and race. It has not been discovered yet why there is a difference between NHW and H women and their risks for developing breast cancer.

**Methods [Study Population]**

New Mexico Sites of 4-Comitas Women’s Health Study (1999-2005)

**Study Objectives**

- Evaluate the association between modifiable risk factors and breast cancer risk in non-Hispanic white and Hispanic women.

**Case Eligibility**

- Hispanic, Native American or NHW ethnically self-reported.

**Control Eligibility**

- Resident of New Mexico

**Ascertainment**

- age ≥ 25 years

**Eligibility**

- women ≤ 5 years randomly selected from driver's license lists

**Methods [HBI Construction]**

Construction of the Healthy Behavior Index

<table>
<thead>
<tr>
<th>HBI Variables</th>
<th>Definitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoking</td>
<td>0 = never smoker; 1 = former; 2 = current</td>
</tr>
<tr>
<td>Alcohol Consumption*</td>
<td>0 = ≤ 0.5 standard drink/day, 1 ≤ 1 standard drink/day, 2 = ≥ 1 standard drink/day</td>
</tr>
<tr>
<td>Waist-Hip Ratio (WHR)</td>
<td>0 = T1: 1.1; T2: 1.2-2.0; T3: ≥ 2.0</td>
</tr>
<tr>
<td>Dietary Pattern</td>
<td>0 = 2XV, 1 = 3XV, 2 = ≥ 4XV</td>
</tr>
<tr>
<td>Vigorous Physical Activity</td>
<td>0 = ≤ 2X, 1 = 3X, 2 = ≥ 4X</td>
</tr>
<tr>
<td>Academy of Nutrition and Dietetics/university/industry/extension</td>
<td></td>
</tr>
</tbody>
</table>

**Methods [Statistical Analysis]**


Descriptive characteristics were compared using chi-square (X2) for significance stratified by case-control status and ethnicity. Associations between healthy behavior index scores and breast cancer risk were calculated with multivariable logistic regression to estimate odds ratios (ORs) with 95% confidence intervals between cases and controls. Multivariable logistic regression determined associations between HBI and risk of breast cancer defined by ER status, compared to controls.

**Results**

Table 1. Description and Prevalence of the Healthy Behavior Index Factors (n=1,031)

<table>
<thead>
<tr>
<th>Factor</th>
<th>NHW</th>
<th>H</th>
<th>NHW</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years ± SD)</td>
<td>54.8±8.9</td>
<td>56.4±7.9</td>
<td>54.8±8.9</td>
<td>56.4±7.9</td>
</tr>
<tr>
<td>Menopausal Status</td>
<td>242 (41.6)</td>
<td>108 (40.1)</td>
<td>242 (41.6)</td>
<td>108 (40.1)</td>
</tr>
<tr>
<td>Parity</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Education</td>
<td>237 (41.6)</td>
<td>111 (41.6)</td>
<td>237 (41.6)</td>
<td>111 (41.6)</td>
</tr>
<tr>
<td>Occupation</td>
<td>237 (41.6)</td>
<td>111 (41.6)</td>
<td>237 (41.6)</td>
<td>111 (41.6)</td>
</tr>
<tr>
<td>Hispanic Smoking</td>
<td>242 (41.6)</td>
<td>108 (40.1)</td>
<td>242 (41.6)</td>
<td>108 (40.1)</td>
</tr>
<tr>
<td>Current</td>
<td>237 (41.6)</td>
<td>111 (41.6)</td>
<td>237 (41.6)</td>
<td>111 (41.6)</td>
</tr>
<tr>
<td>Alcohol Consumption</td>
<td>237 (41.6)</td>
<td>111 (41.6)</td>
<td>237 (41.6)</td>
<td>111 (41.6)</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>25.2±5.1</td>
<td>25.4±5.4</td>
<td>25.2±5.1</td>
<td>25.4±5.4</td>
</tr>
<tr>
<td>Waist Circumference (cm)</td>
<td>53.2±10.3</td>
<td>54.0±10.7</td>
<td>53.2±10.3</td>
<td>54.0±10.7</td>
</tr>
<tr>
<td>Vigorous Physical Activity</td>
<td>237 (41.6)</td>
<td>111 (41.6)</td>
<td>237 (41.6)</td>
<td>111 (41.6)</td>
</tr>
<tr>
<td>No Activity</td>
<td>237 (41.6)</td>
<td>111 (41.6)</td>
<td>237 (41.6)</td>
<td>111 (41.6)</td>
</tr>
<tr>
<td>Diet Pattern</td>
<td>237 (41.6)</td>
<td>111 (41.6)</td>
<td>237 (41.6)</td>
<td>111 (41.6)</td>
</tr>
<tr>
<td>Extensive Receptor</td>
<td>237 (41.6)</td>
<td>111 (41.6)</td>
<td>237 (41.6)</td>
<td>111 (41.6)</td>
</tr>
</tbody>
</table>

**Odds Ratios for Quartiles of the HBI by ER Tumor Status among All Women**

<table>
<thead>
<tr>
<th>Quartiles of Health Behavior Index</th>
<th>Odds Ratios</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00 - 0.33</td>
<td>1.00</td>
<td>1.00 - 1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>0.34 - 0.67</td>
<td>1.21</td>
<td>1.00 - 1.47</td>
<td>0.045</td>
</tr>
<tr>
<td>0.68 - 1.00</td>
<td>1.32</td>
<td>1.10 - 1.59</td>
<td>0.002</td>
</tr>
<tr>
<td>1.01 - 1.33</td>
<td>1.41</td>
<td>1.20 - 1.67</td>
<td>0.000</td>
</tr>
</tbody>
</table>

**Conclusions**

- The HBI was associated with breast cancer risk.
- The association is stronger in non-Hispanic white women, and in women with estrogen receptor positive tumor phenotypes.
- The direction of the association suggests that women with multiple unhealthy behaviors, including drinking, smoking, a poor diet, a high BMI, and WHR, are at greater risk for developing breast cancer.
- Future research may be directed at refining the HBI in replicating these findings in a larger study.
- A policy implication is that a reduction of multiple rather than single risk factors should be addressed in primary interventions.
- Inclusion of risk biomarkers in conjunction with HBI could provide a more sensitive tool for cancer prevention.

**References**

Effects of therapeutic compounds on cadmium-induced prostate cancer

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Abstract
Prostate cancer is the most prevalent form of cancer in men with over 3.3 million existing cases in the U.S. Cadmium is a toxic heavy metal with widespread use in industry, making it a common environmental pollutant. Cadmium exposure induces prostate cancer in humans, as well as in vitro human cell lines and in vivo mouse xenograft models. In previous studies, the natural compound 3, 9-dihydroxy-2-prenylcoumaran [psoralidin (pso)] induced reactive oxygen species in cadmium-transformed prostate epithelial cells (CTPE) to inhibit cancer cell growth and prevent metastasis. It has also been shown to induce both autophagy and apoptosis in these cells. Although these relationships have been established, the molecular processes by which cadmium transformation occurs and pso inhibits cell growth and metastasis are not well defined. To understand these processes, studies on the in vivo transformation of normal human prostate epithelial cells (RPWE-1) into CTPE cells, as well as the molecular effect of pso on CTPE cells, were conducted.

Methods: In this study, we evaluated the change in mRNA expression of epithelial mesenchymal transition (EMT) markers. CTPE cells were exposed to cadmium or pso for 0, 12, 24, 48, and 72 hours, then RNA was extracted and steady state mRNA levels of the EMT markers determined using qRT-PCR.

Hypothesis: We hypothesized that cadmium would induce metastasis by affecting the EMT. Conversely, pso will prevent metastasis in cadmium-transformed cells by inhibiting EMT and lower proliferation by inhibiting autophagy.

Results: In CTPE cell lines, we found two EMT promoting markers, MMP2 and MMP9, showed significantly decreased expression after 24 and 48 hours of pso treatment, p<0.05. Plac8, a marker for autophagy, also showed a significant decrease in expression after 12 hours, p<0.05, but returned to normal levels after 24 hours. Pso-treatment significantly increased expression of E-cadherin, an EMT inhibition marker, at 12 and 24 hours, p<0.01. E-cadherin expression also significantly increased in CTPE xenograft tumor tissue grown in mice treated orally with pso at 24 hours, p<0.001. CTPE tumor tissue also showed a significant decrease in β-catenin expression, an EMT promoter, at 24 hours pso treatment, p<0.01. When CTPE was treated with cadmium, β-catenin and Plac8 expression showed significant increase by 72 hours, p<0.01. Not all markers tested are shown.

Conclusions: Although not all EMT markers tested responded to pso treatment, these results suggest that pso has an inhibitory effect on the EMT and autophagy in cancer cells at the RNA level. This effect prevents metastasis and decreases proliferation by lowering the cancer cell’s protective capacity. Alternatively, cadmium may cause an increase in certain EMT and autophagy markers, leading to cancer cell metastasis and survival.

Future Directions
• Evaluate mRNA expression levels of EMT markers and Plac8 in RWPE-1 cells treated with cadmium.
• Perform Western immunoblots to determine change in protein expression of EMT markers and Plac8 after pso and cadmium treatment.
• Confocal microscopy is needed to evaluate pso and cadmium effect on protein localization, especially for markers which did not significantly inhibit EMT. A change in localization could lead to inactivity without changing expression levels.

Acknowledgements
NCI R25 grant support from University of Louisville Cancer Education Program NIH/NCI (R25- CA134283)
Introduction: The novel small molecule XB05 (1-bromo-1,1-difluoro-nona-2,3-diyl) has been previously documented to selectively target malignant, but not non-malignant cell lines in vitro by disrupting cellular redox homeostasis. The mechanism of action for XB05 is unknown, but in silico and in vitro studies have identified possible roles for XB05 and glutathione redox (GSH) [1]. XB05 is of particular interest because it is a marker for tumor initiating cells (TICs), a subpopulation of tumor cells identified to drive metastasis and recurrence of cancers [2,3]. The purpose of this study was to investigate the effect of XB05 treatment on levels of SOX9 and GR proteins in two cancer cell lines. Additionally, two candidate inhibitors of SOX9 (JT1 and JT2) that were designed to directly target SOX9 activity were examined.

Methods: MDA-MB-231 (breast cancer) and U937 (myeloid leukemia) cell lines were plated at a density of 8.0 x 10^4 cells/T-150 flask and allowed to adhere for 24 hours before treatment with 2 µM XB05, JT1, JT2, or dimethyl sulfoxide (DMSO) as a vehicle control for untreated samples. After treatment for 24 hours, the cell lysates were collected with RIPA buffer containing protease and phosphatase inhibitors and protein concentration was determined. Gel electrophoresis and a subsequent transfer onto a polyvinylidene fluoride membrane were performed on the samples for western blotting using antibodies against SOX9, glutathione reductase and GAPDH (loading control).

Results: Triplet western blot analyses revealed that SOX9 was expressed in the MDA-MB-231 line, but not in the U937 line and that SOX9 protein levels remained similar to the vehicle controls after 24 hour treatment with 2 µM XB05, JT1, or JT2. Both the MDA-MB-231 and U937 lines expressed GR and GAPDH levels remained similar to the vehicle controls after 24 hour treatment with 2 µM XB05.

Conclusions: It appears that although XB05 shows a general preference for SOX9-high cancer cells, there are some exceptions because the U937 cell line (which has high sensitivity to XB05) has very low SOX9 levels. Possibly, XB05 preferentially targets tumor initiating cells (TICs) and SOX9 is a marker for TICs in some cancer types but not in others. Our data do not rule out a role for SOX9 in MDA-MB-231 cells or for GR, but they indicate that SOX9 does not induce changes in protein levels. Further research is required to investigate if the activity of SOX9 or GR is affected by XB05, JT1 and JT2 treatment.

Background: Sex determining region Y box 9 (SOX9) is a transcription factor that appears to induce cell proliferation in some types of cancers (colon, prostate, bladder). Studies suggest that tumors contain a subpopulation of tumor initiating cells (TICs) that express SOX9 and drive cancer progression and metastasis [4,5,6]. Previous research has revealed that SOX9-high cancer cells are more susceptible to the cytotoxic effects of XB05 than non-malignant cells or SOX9-low cancer cells [4,5]. Therefore, we hypothesized that the previously studied molecule, XB05, may induce cytotoxic effects on malignant cells expressing SOX9 via an unknown direct or indirect mechanism.

The cytotoxicity of XB05 in vitro was previously evaluated and described, highlighting the potential application as a synthetic chemotherapeutic drug [4,5,6]. The results of these studies identify the ability of XB05 to induce cell death through apoptotic and non-apoptotic mechanisms in malignant cells. In summary, this molecule interrupts homeostatic defense mechanisms resulting in cytotoxic levels of reactive oxygen species (ROS) and damages in DNA (DSB). Interestingly, a positive correlation exists between SOX9 expression levels and response to XB05 [4]. It has been concluded that SOX9 is expressed in the TICs (cancer stem cells), and that targeting these cells via SOX9 may prevent metastasis and recurrence.

Although the mechanism of XB05 action is unknown, in silico and in vitro studies have identified a possible role for glutathione redox. A virtual screen of proteins that directly bind to XB05 was performed. The results of the virtual screen identified glutathione dehydrogenase as the top protein that is capable of directly binding to XB05. This finding led us to investigate the role of glutathione redox in vitro after XB05 treatment.

Cell culture: MDA-MB-231 (breast adenocarcinoma) and U937 (myeloid leukemia) cell lines were cultured in a humidified incubator at 37°C and 5% CO2. Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies) was used for the MDA-MB-231 cells, and RPMI 1640 media (Life Technologies) was used for the U937 cells. Media was prepared with 10% fetal bovine serum (Life Technologies) and 1% Penicillin/Streptomycin (Life Technologies). XB05, JT1, JT2 compounds were synthesized in the UofL Medicinal Chemistry facility. Cells were plated at a density of 8.0 x 10^4 cells in a T-150 flask (20 ml total volume) and allowed to adhere overnight.

Cell treatments: XB05, JT1, JT2 and vehicle control (DMSO) treatments were initially diluted into media and added to cells to a final concentration of 2 µM for 24 hours.

Preparation of cell lysates: MDA-MB-231 cells were washed with 10 ml ice cold PBS (Life Technologies). Lysates were prepared with RIPA buffer containing phosphatase and protease inhibitors (Millipore). U937 (suspension) cells were pelleted by centrifugation at 1000 g for 5 min. RIPA buffer containing phosphatase and protease inhibitors was added to the pellet. Cell extracts from both lines were centrifuged at 14,000 g at 4°C for 15 min and supernatant was collected. Pierce BCA Protein Assay (Thermo Fisher Scientific) was used according to manufacturer’s directions to determine protein concentration.

References:
3. Trace CF. Unpublished data.
Paracrine Induction of Macrophages by Melanoma Exosomes
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Introduction
Macrophages are key participants in tumor pathogenesis. They can be divided into two general classes (M1 and M2) based on cytokine production. M1-polarized macrophages mediate effective anti-tumor immunity. M2's promote tumor growth via immune suppression. Melanoma tumors and derived factors have been shown to suppress the anti-tumor immune response. Our previous investigations demonstrated that melanoma exosomes prepare lymph nodes for tumor metastasis by creating a pro-angiogenic cancer friendly microenvironment (1, 2). Melanoma exosomes can also inhibit cytotoxic anti-tumor T cells, NK cells and induce MDSCs (3). However, to date, there have been minimal investigations into the direct influence of melanoma exosomes on macrophage function. In this study, we hypothesized that melanoma exosomes might directly induce macrophage M2 polarization.

Significance & Innovation
The role of melanoma exosomes in directly influencing macrophage function is poorly understood. Understanding the ability of melanoma exosomes to influence macrophage mediated pro-tumor processes will further our basic understanding of melanoma pathogenesis. These investigations provide a foundation for the development of novel exosome based therapeutics to antagonize melanoma exosome mediated induction of tumor supportive macrophage functions.

Methods
•Cell Culture: B16F10 melanoma and Raw 264.7 macrophage cell lines were cultured in DMEM with 10% FBS media at 37°C and 5% CO2.
•Exosome Isolation: Exosomes were isolated from B16F10 cell culture media via differential centrifugation. Exosome quantities were measured using a BCA (bicinchoninic acid) assay (Thermo Scientific) to determine protein concentration.
•Treatment: Raw 264.7 cells were added to 96 well plates. After 24 hours, media was aspirated and replaced with one of six treatments: Non-treated (Exosome Free Media), Exosome Treated, LPS treated, IL4 Treated, IL4 + Exosome Treated, and IL4 + Exosome Treated. After 24 hours, supernatants were transferred to be analyzed by ELISA or RT-PCR.
•Cell Viability Assay: PrestoBlue Cell Viability Reagent (Invitrogen) was used to measure the viability and proliferation of non-treated and treated cells.
•Exosome Response: ELISA (Affymetrix) was used to test cytokine production in treated, non-treated and cell supernatants. RT-RT PCR arrays (Qiagen) were used to confirm and extend ELISA results.

Results

M1 Polarization
Exosome Induction of TNF alpha

Figure 1 - TNF-α production in cells treated with LPS, exosomes, and LPS+exosomes. LPS increases the production of TNF-α. Exosome treated cells produced significantly more TNF-α than non-treated cells (p < 3.7e-2). However, LPS + exosome treated cells performed similarly to cells treated with LPS alone. n = 3 independent experiments using pooled batches of exosomes. Error bars = S.E.M. p < 0.05 was considered significant

M2 Polarization
Exosome Induction of TGF beta

Figure 4 - TGF-β production in cells treated with IL4, exosomes, and IL4+exosomes. Treatment with IL4 alone significantly lowers TGF-β production compared to non-treated cells. IL4 and exosomes alone did not lower TGF-β production. n = 3 independent experiments using pooled batches of exosomes. Error bars = S.E.M. p < 0.05 was considered significant

Exosome Induction of IL10

Figure 5 - IL-10 production in cells treated with IL4, exosomes, and IL4+exosomes. Treatment with IL4 alone did not increase the amount of IL10. Exosome treated and IL4-exosome treated cells produced significantly more IL-10 than non-treated and IL4 treated cells. n = 3 independent experiments using pooled batches of exosomes. Error bars = S.E.M. p < 0.05 was considered significant

Conclusions
Here, we hypothesized that melanoma exosomes would directly induce macrophage M2 polarization. Our ELISA findings revealed that melanoma exosomes do not polarize macrophages exclusively as the M1 or M2 direction. Assessment of M1 cytokines revealed that melanoma exosomes significantly increased the production of TNF-α. TNF-α is known to participate in M1 macrophage mediated anti-tumor immunity. However, TNF-α also plays a role in promoting tumor angiogenesis (4). In contrast, IL-18, another M1 derived pro-inflammatory cytokine, was not induced by melanoma exosomes. Induction of standard M2 cytokines revealed similar findings via ELISA. TGF-β was not induced by melanoma exosomes. However, IL-10, the chief M2 cytokine, was significantly expressed.

Additionally, combining exosomes with LPS treatments trended toward increasing TNF-α and IL-18, while combining exosomes with IL-4 decreased TGF-β and significantly increased IL-10 over IL-4 alone.

Results from the RT-RT PCR arrays largely corroborated our ELISA data. However, in contrast to the ELISA results, the PCR data shows that exosomes significantly increased IL-18 mRNA synthesis. Analysis of M2 results revealed, similar to the IL-18 findings, that exosome treated cells contained more TGF-β mRNA despite no difference observed by ELISA. The discrepancy could be a result of assay sensitivity with RT-PCR being more sensitive or may reflect underlying post-transcriptional regulation mechanisms requiring more investigation.

Collectively, these findings suggest that melanoma exosomes induce a mixed macrophage phenotype. For M1, exosomes increase TNF-α. For M2, exosomes increase IL-10. A number of previous studies suggest a complicated relationship between TNF-α and IL-10. TNF-α can induce IL-10 expression (5) or alternatively, IL-10 can suppress TNF-α (6).

Overall, the pattern of macrophage cytokines induced by melanoma exosomes best describes M2b polarization, which is characterized by IL10 expression in the context of typical M1 representative cytokines including TNF-α, IL-18, IL-6 and IL10 (7). M2b response is traditionally associated with humoral immunity, allergic and anti-parasitic immune functions (8). Induction of VEGF-A and Stat3 by melanoma exosomes further suggests polarization toward an M2-like phenotype. This finding is of great importance to our understanding of melanoma exosome mediated tumorigenesis. The results demonstrate that melanoma exosomes can directly polarize macrophages toward a phenotype capable of facilitating pro-tumor supportive angiogenic (TNF-α, VEGF-A) and immunosuppressive (IL-10) functions.

Future Directions: Additional research objectives include validating these investigations using primary mouse and human macrophages. The long term goal is to develop exosomal therapies for melanoma based on macrophage polarization.

Acknowledgements
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References
Imprinting Status of Paternally Imprinted Tandem Genes and their Expression in Ovarian Carcinoma Cell Lines

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1 R2S Cancer Education Program, University of Louisville, Louisville, KY
2 Stem Cell Institute at James Graham Brown Cancer Center, University of Louisville, Louisville, KY

Abstract
I become interested in methylation pattern at differently methylated regions (DMRs) of two paternally imprinted tandem genes (H19/IGF2 and Dlk1-Meg3) a.k.a. GTL2, in human ovarian cancer cell lines (A2780, CAOV3, and OVCAR4) in comparison to healthy tissue (cord blood as control). The methylation status of the H19-IGF2 DMR and Dlk1-MEG3 DMRs was assessed, and so it was the H19-IGF2 and Dlk1-MEG3 ratio of expression. The methods used included combined bisulfite-restriction analysis (COBRA), methylation specific PCR and quantitative real-time PCR. In the first part of this study, no significant changes in methylation of the IGFl2-H19 DMR was observed, but in contrast hypermethylation of the Dlk1-MEG3 DMR in A2780 and OVCAR4 has been noticed, and for all tested ovarian carcinoma cell lines loss of imprinting (LOI) was in the MEG3 DMR. Moreover, lack of correlation between the IGFl2-H19 DMR methylation status and the expression of IGFl2 and H19 genes was found in all ovarian carcinoma cell lines. No direct correlation between the methylation status of the Dlk1-MEG3 DMR and the expression of Dlk1 has been observed with the exception of MEG3. The similar methylation status of the cell lines samples with a different ratio between tandem genes expression suggest that the expression is imprinting independent. Since expression of tandem genes from one locus should be balanced, from lack of expression in the other, the ratio of IGFl2-H19 and of Dlk1-MEG3 was analyzed. When a high level of expression of factors that inhibit cell proliferation was observed in one region a high expression of factors that promote proliferation was observed in the other region. This suggest that both tandem regions complement each other in regulation of cell proliferation.

Objectives
- To examine the methylation status of the Different Methylated Regions (DMRs) in the IGFl2-H19 and Dlk1-MEG3 tandem gene loci.
- To assess the expression of those genes and the ratio of their expression per tandem gene.

Background
Genomic imprinting refers to the process that causes genes to be expressed in a monoallelic and parental-origin-specific manner in a regulated and tissue-dependent fashion. Male-specific alleles of many imprinted genes are preferentially expressed in chromosome, one from each parent, and most autosomal genes are expressed from both maternally and paternally. Genomic imprinting is related to the methylation of cytosine bases in the CGC dinucleotides in the DNA sequence. Almost all imprinted genes have a CpG-rich differentially methylated region (DMR) found to be a key regulator in imprinting gene's expression. The methylation status of imprinted genes is important in cancer studies because it governs the regulation of cell proliferation. Paternally expressed genes generally enhance growth, whereas maternally expressed genes appear to oppose effect. It has been hypothesized that this behavior is the result of different evolutionary pressures (parental conflict hypothesis): the mother tends to distribute resources to all offspring equally and to ensure the fetus would not be too big at the moment of delivery. Father desires to maximize growth and resource acquisition for his offspring alone to favor the fitness of his descendants (Ferguson-Smith, Genomic imprinting: the emergence of an epigenetic paradigm, 2011). Of interest for this study are the tandem genes H19 (maternally expressed) and IGF2 (paternally expressed) found on chromosome 11, and the genes Dlk1 (paternally expressed) and MEG3 (a.k.a. GTL2, maternally expressed) found on chromosome 14 [see Fig. 1]. These two couples need to be in balance for effective development of an organism.

Methods
The methylation analysis was performed on samples from three ovarian carcinoma cell lines – A2780, CAOV3, and OVCAR4 – and on two samples of healthy tissue – cord blood – used as control (see Fig. 2). DNA was isolated from the cells, digested with a specific restriction enzyme kit, and shipped for methylation analysis. After a methylation specific nested polymerase chain reaction (Meth-PCR) the presence of the desired methylation status was detected. Then the products were analyzed by combined bisulfite-restriction analysis (COBRA) (see Fig. 3) followed by densitometric analysis. This second part of the study regarded the expression of the genes. cDNA was isolated from samples of the cell lines and converted into cDNA by reverse transcription. The obtained cDNA was used as a template for the final step of the analysis of the expression of the genes by quantitative real-time PCR (see Fig. 4).

Summary of Results
- There are no significant changes in methylation status of the IGFl2-H19 DMR.
- DLK1-MEG3 DMR is hypermethylated in A2780 and cell lines.
- Lack of imprinting (LOI) in the MEG3 DMR occurs in all tested ovarian cancer lines.
- Lack of correlation between the methylation status of the DLK1 DMR and the expression of IGFl2 and H19 genes was observed in all ovarian carcinoma cell lines.
- There is no direct correlation between the methylation status of the DLK1 DMR and the expression of DLK1 gene in all ovarian carcinoma cell lines samples.
- In contrast MEG3 expression is observed to be higher in CAOV3 cell line in comparison with A2780 and OVCAR4 cell lines. This correlates with the differences in methylation of DLK1-MEG3 DMR between analyzed ovarian carcinoma cell lines.
- The similar methylation status of the cell lines samples with a different ratio between tandem genes expression suggest that the expression is imprinting independent.

Conclusions
- Analysis of IGFl2/H19 and DLK1/MEG3 ratio of expression indicates a shift toward factors that inhibit cell proliferation in one tandem gene region, compensated by factors that promote proliferation encoded in the other region. This suggest that both tandem regions complement each other in regulation of cell proliferation. Changes in the methylation of DMRs in DLK1-MEG3 cluster suggest that the genes encoded by this specific region might be relevant in the regulation of cell proliferation.

Future studies
- Future studies on IGFl2/H19 and DLK1-MEG3 DMRs methylation will be performed using samples taken from human primary tumors to confirm the results so far obtained.
- Since this study suggest imprinting independent expression of IGFl2 and H19, the analysis of the type of expression, monoallelic vs biallelic, will be performed.
- Changes in the methylation of DMRs in DLK1-MEG3 cluster suggest that the genes encoded by this specific region might be relevant in the regulation of cell proliferation. Therefore more detailed studies will be performed by targeting the expression of these genes by transfecting the cells with appropriate plasmids to achieve expression of DLK1 or MEG3, and plasmids encoding sIVA to downregulate DLK1 or MEG3 levels.

Acknowledgments
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The Effect of G-quadruplex Oligonucleotide Sequences Targeting c-MYC, SOX2 and H-TERT in Melanoma Cell Lines

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Abstract/Introduction

Malignant melanoma is the deadliest form of skin cancer and it is estimated that 10,310 people will die in the US of melanoma in 2016[1], SPQ1, SQX2, CEX, H-TERT, and H-MYC are a line of many genes expressed in melanoma. This study focuses on c-MYC, SOX2, and H-TERT. The c-MYC gene is a master regulator that is critically involved in the regulation of many gene promoters: a novel anticancer strategy?

Results

Figures 1: Evaluation of the basic c-MYC, SOX2, and h-TERT gene expression in melanoma cell line relative to HS27 fibroblasts.

Results/Discussion

Figures 2: Protein Expression of c-MYC, SOX2, and h-TERT genes.

Figures 3: Effect of G-quadruplex oligonucleotide treatment for 6 days on cell proliferation.

Figures 4: SOX2, c-MYC, and h-TERT gene expression on cell lines (A375, Mel2, and HS27) treated with Pu27, Pu3+ or Tert+1 oligonucleotides for 6 days.

Materials & Methods

Dilutions of Pu27 to target the c-MYC gene. Pu3+ to target SOX2 gene, tetr+1, Tert+1 Tert+4/6 to target h-TERT gene.

Cell Culture: Four melanoma cell lines and one normal human skin fibroblasts, A375, SK-Mel2, SK-Mel3, and HS27 (human normal fibroblast), were cultured and transfected in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum and 2% Penicillin/Streptomycin.

MTT Assay: A375, SK-Mel2, SK-Mel3, and HS27 were seeded in 96-well flat bottom plates at 3 x 103 cells/well in 150 µl. Plated cells were treated with doses of 5 and 50µM of Pu27, Pu3+, tert+1 and tert+4/6 for 4 days. Cell proliferation was measured by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), MTT was used as blank and measured using 540 nm Microplate reader and Spectrophotometer to read absorbance at 570nm.

Cell Treatment for gene expression in all (A375), cell line (SK-Mel2, SK-Mel3, and HS27) and the control cell line (Mel2) were plated at 2 x 103 cells/well in 1 well plates and treated with 4µl of Pu27, Pu3+ or tert+1 for 4 days. RNA was collected and gene expression was measured.

Gene Expression: The basic gene and protein expression of SK-Mel2, SK-Mel3, and HS27 were determined in A375, Mel2, SK-Mel2, SK-Mel3, and HS27 cell lines. The effect of oligonucleotide treatment (control and tert+1 and tert+4/6) was evaluated in A375, Mel2, and HS27 was determined using:

QRT-PCR: Rheol magnol was used to extract RNA. cDNA was synthesized from the extracted RNA using SuperScript II (Invitrogen DNA Synthesis). Quantitative Real Time PCR was performed with primers pair for SOX2, c-MYC, h-TERT and GAPDH as a housekeeping control.

Western Blotting: Protein lysate samples were used using Affinity Mammalian Protein Extraction Reagent with Protein Inhibitor. Proteins were separated based on size of protein using SDS-Mai gel electrophoresis. For determination the antibodies were used: rabbit anti-SOX2, followed by anti-rabbit-HRP; mouse anti-c-MYC, followed by anti mouse HRP; and rabbit anti-Actin, followed by anti-rabbit HRP. The presence of the bands was revealed using chemiluminescence through development on X-ray films.

Conclusion

Most cell lines were sensitive to Pu27 oligonucleotides that target c-MYC and to the different oligonucleotides targeting SOX2 and H-TERT. The use of G-quadruplex forming oligonucleotides to target specific genes such as c-MYC, SOX2 or h-TERT could have therapeutic application in melanoma.

This study suggests that the presence of mutations within h-TERT promoter region of A375 may affect these cell responses to oligonucleotides targeting the same region.

Future Directions: Correlation of the sensitivity of the cell lines to the h-TERT oligonucleotides and the presence of mutations in the promoter region of h-TERT. Exclusion of mechanism of action of h-TERT oligonucleotides and determine molecular pathways in melanomas.

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References


The effect of the oligonucleotides on cell proliferation by MTT assay: (Fig. 3) A375 showed the best growth inhibition after treatment with all of the oligonucleotides targeting c-MYC. tert+1 had the best effect on cell inhibition compared to the other two oligonucleotides targeting SOX2 and H-TERT. All cell lines showed some level of growth after treatment with Pu27, Pu3+ and tert+1.

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**Introduction**

- Current chemotherapeutic treatments for non-small cell lung cancers (NSCLC) can be made more effective through the use of oral supplements.
- β-glucan is a naturally occurring product derived from yeast that has been shown to have a substantial effect on reducing tumor progression and inducing a cytotoxic response within the immune system.
- Whole β-glucan particles (WGP) have been shown to reduce the frequency of myeloid derived suppressor cells (MDSC) in peripheral blood through apoptosis.
- MDSCs are undifferentiated myeloid cells that are generated by the secretion of cytokines by cancerous cells. Unlike most myeloid cells, MDSCs have immunosuppressive properties rather than immunosupportive properties.
- The James Graham Brown Cancer Center is conducting an ongoing clinical trial that treats NSCLC patients that haven’t received chemotherapy or radiation for at least six months with WGP taken orally for 10-14 days, collecting blood before and after WGP treatment.
- There is currently no data on how demographic variables affect the response of patients to WGP treatment. This study aims to match demographic variables with percent change of MDSC frequency to examine potential trends that statistically show a certain sub-population responds better to WGP treatment.

**Methods**

- To obtain demographic information of the patients in this study, past medical records were reviewed and specific information was recorded for six categories: gender, age, race, smoking status (calculated in pack years), staging, and histology.
- Using whole blood samples from patients before and after WGP treatment, MDSC frequency in peripheral blood was analyzed using flow cytometry.
- After compiling the lab and clinical data, a spreadsheet was created and analyzed using the SAS statistical program utilizing a two sample t-test to make a determination on any statistical significance between demographic factors and the percent change of MDSCs before and after WGP treatment.

**Results**

This table summarizes the mean percent change differences of MDSCs among sub-populations. As expected, WGP lowered the MDSC percentage among all groups and sub-populations. Although there is some variation within groups, there was found to be no statistically significant difference in the decrease of MDSCs between any of the sub-populations.

**Conclusions**

Despite the fact there were no statistically significant differences found in any of the groups, the data shows some interesting trends between sub-populations when examining mean percentage change. This is especially true when looking at pack years; notice how the difference between the mean percent changes of MDSCs are much greater than any other group analyzed. However, this study simply serves as a pilot analysis that is hypothesis generating to target certain sub-populations for further investigation. More research needs to be conducted to draw any definitive conclusions.

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**References**