The Interrelationship of BRCA1 185delAG, Interleukin-1β, and Ovarian Oncogenesis

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The Interrelationship of BRCA1 185delAG, Interleukin-1β, and Ovarian Oncogenesis

by

Kamisha T. Woolery

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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College of Medicine
University of South Florida

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Date of Approval:
June 27, 2014

Keywords: ovarian cancer, BRAT, inflammation, ovarian surface epithelium, malignant transformation

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Dedication

This dissertation is dedicated to my wonderful husband, who without his love, patience, and support I could not have endeavored to its completion; I love you. I love you my wonderful daughter, my greatest accomplishment. I would also like to thank my mother who always believed in me. I would like to thank Dr. Kruk, my mentor, for teaching me to think like a scientist and to celebrate every victory. I could not have completed this dissertation without you supporting me, challenging me, and never giving up on me. Lastly, I would like to thank my lab mates and peers, Rebecca, Nicole, Kendra, Nadine, and Kim; I could not have survived without your encouragement and friendship.
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List of Abbreviations

3D       3-Dimensional
AATD  α1-Antitrypsin deficiency
APC/C     Anaphase-promoting complex/cyclosome
ASC    Apoptosis-associated speck-like protein containing a C-terminal caspase-recruitment domain
ASRM  American Society of Reproductive Medicine
ATM     Ataxia telangiectasia mutated
BARD1  BRCA1-associated RING domain protein 1
BLAST  Basic local alignment search tool
BMI      Body mass index
BRAT  BRCA1 185delAG Amino Terminal truncated protein
BRCA  Breast cancer susceptibility gene
BRCA1  Breast cancer susceptibility gene 1
BRCA2  Breast cancer susceptibility gene 2
BRCT  BRCA1 C terminus
CA125  Cancer antigen 125
CBP     CREB-binding protein
cDNA    complementary DNA
ChIP  Chromatin immunoprecipitation
cIAP1  Cellular inhibitor of apoptosis 1
CID     Chronic inflammatory disease
COPD  Chronic obstructive pulmonary disease
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>CREB</td>
<td>Cyclic AMP response element (CRE)-binding protein</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>Ct</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>EOC</td>
<td>Epithelial ovarian cancer</td>
</tr>
<tr>
<td>FAP</td>
<td>Fibroblast activation protein</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FH</td>
<td>Family history</td>
</tr>
<tr>
<td>FIGO</td>
<td>International Federation of Gynecology and Obstetrics</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HMGA2</td>
<td>High mobility group A2</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IDP</td>
<td>Intrinsically disordered protein</td>
</tr>
<tr>
<td>IDR</td>
<td>Intrinsically disordered region</td>
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<tr>
<td>IL-12</td>
<td>Interleukin-12</td>
</tr>
<tr>
<td>IL-13</td>
<td>Interleukin-13</td>
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<tr>
<td>IL-16</td>
<td>Interleukin-16</td>
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<tr>
<td>IL-18</td>
<td>Interleukin-18</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>Interleukin-1 receptor antagonist</td>
</tr>
</tbody>
</table>
IL-1α  Interleukin-1α
IL-1β  Interleukin-1β
IL-2   Interleukin-2
IL-4   Interleukin-4
IL-6   Interleukin-6
IL-8   Interleukin-8
iNOS  Inducible nitric oxide synthase
IOSE  SV-40-Large-T antigen transfection OSE
IP    Immunoprecipitation
IPTG  Isopropyl β-D-1-thiogalactopyranoside
IRB   Institutional review board
LH    Luteinizing hormone
MCC/USF Moffitt Cancer Center at the University of South Florida
MET   Mesenchymal-epithelial transitions
MMP-1 Matrix metalloproteinase-1
MMP-9 Matrix metalloproteinase-9
mRNA  Messenger RNA
NALP3 NACHT, LRR and PYD domains-containing protein 3
NCBI  National Center for Biotechnology Information
NES   Nuclear export signal
NFH   No family history
NF-κB Nuclear factor kappa-light-chain-enhancer of activated B cells
NLS   Nuclear localization signal
NO-NSAID Nitric oxide-releasing NSAIDS
NSAID Non-steroidal anti-inflammatory drug
OC    Ovarian cancer
OSE   Ovarian surface epithelium
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>RING</td>
<td>Really interesting new gene</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RQ</td>
<td>Relative mean mRNA expression</td>
</tr>
<tr>
<td>SCD</td>
<td>SQ-cluster domain</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming growth factor beta 1</td>
</tr>
<tr>
<td>TGH</td>
<td>Tampa General Hospital</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumor protein p53</td>
</tr>
<tr>
<td>TVS</td>
<td>Transvaginal ultrasonography</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>wtBRCA1</td>
<td>Wild-type BRCA1</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
</tr>
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</table>
Abstract

While the etiology of ovarian cancer (OC) is not completely understood, evidence suggests that chronic inflammation may promote malignant transformation. However, familial history remains the strongest risk factor for developing OC and is associated with germline BRCA1 mutations, such as the 185delAG mutation. Normal human ovarian surface epithelial cells expressing the 185delAG mutant, BRAT, exhibit molecular and pathological changes that may contribute to OC oncogenesis. In the current study, I sought to determine whether BRAT could promote an inflammatory phenotype by investigating BRAT’s impact on the expression of the proinflammatory cytokine, Interleukin-1β (IL-1β). Using a culture model system of normal human ovarian surface epithelial (OSE) cells with and without the BRCA1 185delAG frameshift mutation, BRAT, I investigated BRAT’s role in IL-1β expression. OSE cells stably expressing the 185delAG mutation and ovarian surface epithelial cells with endogenous 185delAG were analyzed for differential target gene expression by real time PCR, western blot, ELISA, luciferase reporter and siRNA assays. Normal and malignant breast epithelial cell lines transiently expressing BRAT were also evaluated by real time PCR to determine whether BRAT-induced IL-1β expression is tissue specific. BRAT-expressing OSE cells exhibited enhanced IL-1β mRNA and protein expression. However, expression of BRAT in all breast cell lines failed to significantly alter IL-1β expression levels so that BRAT-mediated IL-1β expression promoting a chronic inflammatory phenotype conducive to malignant transformation may be limited to the ovary. Secondly, since OSE cells
expressing the BRCA1 185delAG mutation have increased levels of IL-1β that may contribute to malignant transformation, in a pilot study, I sought to assess whether elevated urinary levels of IL-1β are associated with OC as well as compare urinary IL-1β levels with clinical parameters. Urinary and serum levels of IL-1β were analyzed by ELISA and biostatistical analysis from a patient cohort consisting of healthy women (N=10), women with ovarian benign disease (N=23), women with OC (N=32), women with other benign gynecological conditions (N=22), and women with other gynecological cancers (N=6). Urinary IL-1β levels were elevated in patients with ovarian benign disease and a first degree family history of ovarian and/or breast cancer. Urinary IL-1β levels were also correlated with increased body mass index. Urinary and serum IL-1β levels were increased in ovarian benign and OC patient samples supporting the theory of elevated urinary IL-1β being associated with cancer progression. Lastly, I sought to begin early molecular characterization of BRCA1 185delAG to better understand its role in ovarian transformation. I isolated 185delAG protein expressed in E. coli and utilized web tools to analyze the amino acid sequence to determine the molecular and structural characteristics. The study results showed the predicted BRCA1 185delAG protein product is an ordered, self-aggregating, alpha helical protein structurally and molecularly distinct from wild-type BRCA1. The BRCA1 185delAG amino acid sequence contained domains with resemblance to the Peptidase M20 family. Isolation of the BRCA1 185delAG protein product will allow for further protein analysis to better understand its’ oncogenic functions; as well as, elucidate the mechanism of tissue-specific BRAT-mediated IL-1β expression since increased IL-1β expression may represent an early step contributing to OC.
Chapter 1:

Background

Overview of Ovarian Cancer

Ovarian cancer (OC) is the fifth leading cause of cancer death among women after lung, breast, colorectal, and pancreatic cancer [1]. It is estimated that 22,240 new cases will be diagnosed in the U.S. and that 14,000 women die annually from this disease [2]. The one year survival rate for OC can be as high as 79% [3]; and when diagnosed in an early stage the 5-year survival rate is 92% [1]. However, when diagnosed at a later stage, the 5-year survival is generally no better than 27% [1]. The high mortality associated with this disease is generally attributed to the fact that most cases typically present in late stage when OC has already spread beyond the ovary, that the disease is generally asymptomatic in early stages, and that an effective screening test is lacking [4, 5]. In addition to involving the ovary, ovarian epithelial cancer cells are shed into intraperitoneal ascites fluid and easily disseminate throughout the peritoneal cavity with preferential metastasis to the omentum and peritoneum as well as other local organs. However, familial history is the strongest risk factor for the development of OC and the majority of hereditary OCs are associated with mutation of the tumor suppressor breast cancer susceptibility gene 1 (BRCA1) [6]. Carriers of the BRCA1 mutation have a 30% risk of developing OC during their lifetime [7].
Clinical Management of Ovarian Cancer

OC is most often diagnosed in late stage due to nonspecific symptoms in early stages and a lack of adequate screening methodologies. OC is symptomatic even in early stages with 89% of stage I & II patients reporting bloating, gas pains, pelvic discomfort, and urinary frequency [8]. However, these symptoms can often be confused with other more common disorders or can be attributed to benign gastrointestinal, genitourinary, musculoskeletal, or gynecologic disorders [8]. Furthermore, even when presenting with such symptoms, there is a lack of adequate screening methodologies. Current detection methods include serum tumor markers such as CA125, imaging such as transvaginal ultrasound, and physical examination [9]. To date, the best screening method is a combination of serum CA125 levels and transvaginal ultrasound [10]; however, CA125 levels are still only elevated in 50-60% of sera from patients with stage I disease [11].

Optimal cytoreduction followed by platinum based chemotherapy remains the mainstay of therapy in the management of advanced epithelial OCs [12]. However, while the response rate to primary chemotherapy can be as high as 76%, response rate is dramatically reduced after relapse of disease [13]. Platinum resistance, defined as disease recurrence less than six months from completion of therapy is an important prognostic predictor. Patients with platinum-resistant tumors have a response rate of less than 10% when retreated with platinum compounds [14, 15] and alternative options also have poor response rates of 18-30% [16-23].

Etiology of Ovarian Cancer

The majority of OCs are sporadic in origin, but about 10% of all epithelial ovarian carcinomas are associated with a hereditary predisposition and are characterized by an increased
incidence and earlier onset of disease [24]. Epidemiological studies suggest that, besides race [25] and familial history of breast or OC [26-28], events associated with ovarian surface epithelium (OSE) traumatization may result in aberrant OSE growth leading to ovarian epithelial carcinogenesis [29]. Thus, increased age, reproductive history (nulliparity), early menarche, late menopause and fertility drug use increase the risk for OC. In contrast, suppression of ovulation by pregnancy, lactation or oral contraceptive use decrease the risk for OC. Lifestyle factors including dietary fat intake and smoking may also increase the risk for OC while dietary intake of vitamins A, C, D and E may protect against OC [4, 30, 31].

Consequently, there are four traditional hypotheses on the etiology of OC. (1) Incessant ovulation: damage during ovulation and repair makes the OSE more susceptible to errors in DNA replication. Decreased number of ovulation cycles resulting from long term oral contraceptive use, high parity, and lactation decreases the risk of OC; while early menarche and late menopause are risk factors for developing OC (Reviewed in [32]). (2) Gonadotropin stimulation: hormones FSH and LH promote growth, and increased cell divisions and mutations; furthermore, FSH upregulates oncogenes (Reviewed in [32]). However, this has become more controversial as studies show no relationship between circulating FSH and OC risk in postmenopausal women [33]; as well as, reduction of OC risk with higher circulating FSH [34]. (3) Hormonal stimulation: high concentrations of androgens in the tumor microenvironment promote carcinogenesis, while progestins decrease the risk of OC. Polycystic ovarian syndrome which results in high levels of circulating androgens increases the risk of OC and progestin use induces OSE cell apoptosis (Reviewed in [32]). (4) Inflammation: damaged OSE induces inflammation and promotes reconstruction and mutation susceptibility (Reviewed in [32]). Events promoting chronic inflammation in the pelvic region appear to promote malignant
transformation [35-39], while non-aspirin non-steroidal anti-inflammatory drugs (NSAIDs) may be protective against OC [40-43].

Since OC is a heterogeneous disease composed of different tumor types with differing pathological features and behaviors, a dualistic model has additionally been proposed to categorize OC tumors based upon morphological and molecular genetic studies into two groups: type I and type II [44-46]. Type I tumors are comprised of low-grade serous carcinomas, low-grade endometrioid, clear cell, and mucinous carcinomas. These low-grade tumors develop in a stepwise manner from precursor lesions, such as borderline tumors and endometriosis, to malignancy. They usually are confined to the ovary (stage 1) and have a good prognosis. Type I tumors are more genetically stable and typically display a variety of somatic mutations that include \textit{KRAS}, \textit{BRAF}, and \textit{PTEN} but very rarely \textit{TP53}. Type II tumors are highly aggressive and most often present in advanced stage (stage III and IV) resulting in a poor outcome. Type II tumors account for approximately 75% of all epithelial OCs and are diagnosed as high-grade serous, high-grade endometrioid, malignant mixed mesodermal tumors, and undifferentiated carcinomas. They are chromosomally unstable and have \textit{TP53} mutations in more than 95% of cases; and rarely display the mutations found in type I tumors. Type II tumors have BRCA inactivation, either by mutation or promoter methylation, which occurs in up to 40% to 50% of high-grade serous carcinomas. Inactivation of BRCA has not been reported in type I tumors.

**Ovarian Cancer Cell Origin**

Over 85% of OCs arise from and/or involve the OSE [12, 47]. Due to the common embryonic origin of OSE with the epithelia lining fallopian tube, endometrium and cervix, histologic subtypes of OSE-derived tumors include serous, endometrioid, and mucinous tumors,
respectively. Likewise, the expression of stromal characteristics by OSE cells may be related to their shared mesodermal origin from the coelomic epithelium and to their close developmental relationship to ovarian stromal fibroblasts. For example, OSE express both vimentin and keratin intermediate filaments characteristic of connective tissue and epithelium, respectively [48]. Further, when maintained on collagen type I gel, OSE cells undergo a modulation into a spindle-like morphology which accompanies collagen gel contraction [49]. In contrast, OSE cells invade Matrigel and can remodel extracellular matrix (ECM) by secretion of collagenase, gelatinase and stromelysin as well as produce laminin and collagens I, III and IV [50, 51]. Consequently, the interaction with stroma and the ability of OSE to produce, lyse and reconstruct ECM indicates that OSE has the capacity to undergo epithelial-mesenchymal conversions which may not only be necessary for restoring the integrity of the OSE following ovulatory damage, but may also contribute to invasion and metastasis with malignant transformation of the OSE.

However, more recently the epithelial lining of the fallopian tube fimbriae has been implicated as the site of OC origin and the OSE as a secondary site of metastasis [45]. Several studies of prophylactic salpingo-oophorectomy in BRCA mutation carriers have found the earliest forms of serous carcinomas more frequently in the fallopian tube than in the ovary [52-54]. Non-malignant fallopian tube epithelium collected in the luteal stage of the menstrual cycle, when the epithelium is exposed to follicular fluid after ovulation, was molecularly similar to high grade serous OC in BRCA mutation carriers [55]. Furthermore, gene expression profiles indicated an increased expression of proinflammatory genes, including interleukin-8 (IL-8) and tumor-promoting NF-κB target genes [56]. In confirmation, an ex vivo culture model of bovine fallopian tube epithelium exposed to human follicular fluid demonstrated increased expression of inflammation genes [57].
An all-encompassing theory now proposes that the adnexal peritoneum, fallopian tubal epithelium, and OSE be viewed as a single and central unit for ovarian oncogenesis since these structures all originate in the mesodermally-derived embryonic coelomic epithelium [58]. Epithelial transitions between sites represent areas with enhanced susceptibility toward neoplasia [59]. In support, other epithelial transitions that play a role in neoplasia include the cervical squamocolumnar [60], gastroesophageal [61], and anorectal junctions [62]. By immunohistochemistry, Seidman, et. al. showed evidence of transitional cell metaplasia, chronic inflammation, and mesothelial hyperplasia at the fallopian tube-peritoneal junction (tuboperitoneal junction) where serous epithelium meets the peritoneal mesothelium [63]. They also suggested similar epithelial transitions may be located where tubal-type epithelium borders mesothelium in OSE inclusions and in the cell adhesions on the ovarian surface that form gland-like and cystic spaces lined by peritoneum and serous epithelium [63]. Kuhn, et. al. suggested benign Brenner tumors may arise from the transitional metaplasia of the tuboperitoneal junction and seed to the ovary [64]. Lastly, epithelial transitions may host novel stem cell niches for OSE regeneration that are prone to malignant transformation [65].

**Inflammation and Ovarian Cancer**

During tissue repair following ovulation, OSE undergoes epithelial-mesenchymal transition (EMT) in response to its local microenvironment assuming a fibroblast-like phenotype which promotes OSE migration, proliferation and matrix remodeling. ‘Incessant ovulation’ proposes that repeated injury to the OSE with subsequent rounds of cellular proliferation can not only lead to the acquisition of replicative DNA errors contributing to genetic instability and ensuing malignant transformation [66], but also to chronic inflammation [67]. Such
inflammation is associated with oxidative stress and recruitment of activated immune cells, including macrophages, T cells, B cells, natural killer cells, neutrophils, and granulocytes [68] resulting in a locally marked increase in cytokines/chemokines (interleukins and tumor necrosis factors) and matrix-remodeling enzymes (plasminogen activators and collagenases) that can also promote tumor formation [66]. In addition to presenting as an inflammatory disease, inflammation of the ovarian epithelium has been associated with increased risk for OC [67, 69-71] and endometriosis is associated with risk for clear cell carcinoma of the ovary [72-76]. Consequently, the potential clinical contribution of inflammatory mediators, including interleukin-6 (IL-6) and IL-1, for ovarian tumor initiation and progression should not be overlooked (summarized in Table 1.1).

**Interleukin-6**

IL-6 is a pleiotropic cytokine that plays a major role in the immune system in response to injury and infection, as well as, inflammation [77]. IL-6 is produced by T cells, monocytes, fibroblasts, endothelial cells, and keratinocytes [78]. Depending on the experimental system, IL-6 has been suggested to have both proinflammatory and anti-inflammatory properties *in vitro* and *in vivo* [79]. However, it appears that IL-6 acts predominately as an anti-inflammatory and immunosuppressive cytokine by directly suppressing IL-1 and tumor necrosis factor-α (TNF-α), inducing release of glucocorticoids, and inducing natural antagonists to IL-1 and TNF-α [79]. In an *in vitro* prostate cancer model, cancer-associated fibroblasts secreted higher amounts of IL-6 than normal fibroblasts which resulted in an increased proliferation of normal epithelial cells and increased endothelial cell migration towards the cancer-associated fibroblasts and/or their conditioned media through transwell migration assay [80]. It is not surprising then that Spaeth et
Table 1.1 Proposed Functions of IL-6 and IL-1 in OC

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Inflammatory Function</th>
<th>Proposed Function in OC</th>
<th>Model</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>Proinflammatory</td>
<td>• Stimulate angiogenesis • Recruit immunocompetent cells • Increase platelet count</td>
<td>In vitro/In vivo • In vitro/In vivo • Phase I clinical trial</td>
<td>[81] [82-87] [88, 89]</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Proinflammatory</td>
<td>• Increase expression of MMP-1 • Stimulate production of pro-angiogenic factors • Promote OC cell migration &amp; invasion</td>
<td>In vitro • In vitro • In vitro</td>
<td>[90] [91] [92]</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>Anti-inflammatory</td>
<td>• Antagonist to IL-1α &amp; IL-1β • Abundant in the OC ascites</td>
<td>In vivo</td>
<td>[93] [84, 94]</td>
</tr>
<tr>
<td>IL-6</td>
<td>Anti-inflammatory</td>
<td>• Promote OC cell proliferation • Stimulate angiogenesis • Enhance endothelial cell migration • Increase OC cell lines capacity to secrete MMP-9 • Increase chemotactic and/or chemokinetic activity • Increase invasiveness • Induce chemo-resistance • Increase platelet count</td>
<td>In vitro • In vitro/In vivo • In vitro • In vitro • In vitro • In vitro • Phase I &amp; II clinical trial</td>
<td>[95] [96] [96] [97] [98] [98] [97, 99, 100] [101, 102]</td>
</tr>
</tbody>
</table>
al. found that tumor associated fibroblasts derived and differentiated from mesenchymal stromal stem cells produced and secreted IL-6 that similarly promoted OC cell proliferation [95].

In the normal ovary, aside from production by activated stromal immune cells, IL-6 is produced by granulosa cells [104] and OSE cells [105, 106]. However, neoplastic ovarian cells also routinely overexpress IL-6 in vitro [105-107] and greater amounts of IL-6 are present in the cystic fluid of malignant tumors when compared to cystic fluid of benign tumors [78]. Likewise, OC ascites are rich in IL-6 [99, 108-110]. Consequently, elevated levels of IL-6 in the blood and ascites are associated with poor prognosis in OC [111].

While the role of IL-6 in the etiology of OC is not fully understood, we might predict that IL-6 contributes to OC by promoting angiogenesis, tumor invasion, and chemoresistance. Specifically, in vivo treatment with IL-6 induced angiogenesis and enhanced endothelial cell-mediated migration of human ovarian carcinoma cells [96]. IL-6 may also be involved in the tumorigenic processes of OC cell lines by increasing their capacity to secrete matrix metalloproteinase-9 [97]. Further, OC cell lines cultured with IL-6 demonstrate increased chemotactic and/or chemokinetic activity and increased overall invasiveness [98]. Overexpression of IL-6 is also associated with chemoresistance in OC cells [99, 100]. In vitro studies of OC cell lines further show that autocrine production of IL-6 decreased their responsiveness to cisplatin and paclitaxel [77]. Interestingly, elevated serum IL-6 levels are uniquely associated with OC compared to other gynecological malignancies and although this is a much less sensitive biomarker for OC than CA125 [99], it may still serve as a useful prognostic indicator of disease aggressiveness and/or responsiveness to chemotherapy.
Interleukin-1

There are 11 members of the IL-1 cytokine family that have proinflammatory or anti-inflammatory activity. The most thoroughly studied cytokines from this family are two agonist cytokines: IL-1α & IL-1β, and one antagonist cytokine: interleukin-1 receptor antagonist (IL-1Ra). Though all of these cytokines are associated with chronic inflammatory diseases (CIDs), IL-1β appears to be the primary mediator of inflammation in CID [93].

IL-1β is synthesized in a precursor form as a 31kD protein that is cleaved by caspase-1 into its active 17kD mature secreted form [112]. IL-1β is mainly produced by monocytes and macrophages, but can also be produced by endothelial cells, fibroblasts, and epidermal cells in response to bacterial or innate immunity stimulation [113]. Both normal and malignant epithelial ovarian cells also produce IL-1 [111], although activated immune cells in the stroma remain the major source of IL-1 [114]. Constitutive production of IL-1β by ovarian carcinoma cells [115] enhances their invasion capacities by increasing expression of matrix metalloproteinase-1 [90] and stimulating production of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) [91]. Additionally, fibroblasts cultured with conditioned media from a highly metastatic OC cell line or with IL-1β induced transformation of the fibroblasts to myofibroblasts expressing α-smooth muscle actin (α-SMA) [92]. This coincided with induction of myofibroblast expression of fibroblast activation protein (FAP), a cell-surface serine protease with capacity to remodel ECM by cleaving collagen type 1 [92], thereby promoting OC cell migration and invasion.

IL-1α is likewise synthesized in a precursor form as a 31kD protein that is cleaved by Ca²⁺-dependent protease calpain into an active 17kD mature cell-associated form [87, 112]. IL-1α is produced by monocytes, macrophages, epithelial cells, keratinocytes, and fibroblasts [116]. However, because it remains mainly cell-associated, IL-1α induces less inflammation and
angiogenesis than IL-1β [81]. Further, since IL-1α is generally secreted to a lesser extent than IL-1β, it is not commonly detected in bodily fluids except when released from necrotizing cells in cases of severe inflammation [87]. In its cell-associated form, IL-1α mainly acts as a tumor suppressor in malignant cells by recruiting immunocompetent cells to the tumor microenvironment and assisting in an immunologic response to combat tumor growth [82-84, 87]. IL-1α tumor suppressor characteristics have been further confirmed in vivo where IL-1α produced by tumorigenic fibroblasts decreased the number of tumor growths, as well as increased tumor rejection by facilitating activation and expansion of helper T cells [85, 86]. Consequently, in OC, IL-1β generally promotes invasiveness, tumor angiogenesis, and induces immune suppression while IL-1α reduces tumorigenecity by inducing antitumor immunity [117].

Lastly, IL-1Ra is an antagonist that inhibits IL-1α and IL-1β from binding to both IL-1 receptors, but does not activate IL-1 receptor cell signaling cascades. IL-1Ra is secreted by the same cell types as IL-1α and IL-1β including, monocytes, macrophages, and OSE [118]. Further, high levels of IL-1Ra in cultured tumor-derived macrophages compared to cultured tumor-derived ovarian epithelial cells [119] suggest that the major contributors of IL-1Ra are activated macrophages. IL-1Ra is excessively abundant in the ascites of late-stage OC patients compared to IL-1Ra levels in ascites and/or serum levels from early-stage OC and benign ovarian tumors [94]. Higher levels of IL-1Ra in ascites and/or serum appear associated with poor prognosis and reduced overall survival [94]. Since IL-1Ra does not activate IL-1 receptor cell signaling cascades and IL-1Ra production can be induced by IL-1β and/or IL-1α, it is plausible that the increased IL-1Ra in the ascites and the associated poor prognosis is due to secreted IL-1β signaling [84]. In agreement, Mustea et. al. found that increased levels of IL-1β in the serum and/or ascites was associated with decreased survival [94].
Interestingly both IL-6 and IL-1 levels are elevated in ascites. *In vitro* studies show that treatment of granulosa cells with IL-1 increases IL-6 production in a dose-dependent manner [104] and, likewise, treatment of OC cell lines with IL-1β, led to increased secretion of IL-6 [120]. Consequently, the contribution of IL-1 to initiate and promote OC progression by regulating IL-6 expression deserves consideration.

**BRCA1**

Familial history is the strongest risk factor for the development of OC and the majority of hereditary OCs are associated with mutation of the tumor suppressor breast cancer susceptibility gene 1 (BRCA1) [6]. Women with inherited mutations of BRCA1 have a 40-60% lifetime risk of OC and 11-27% lifetime risk of breast cancer [121-125]. The *BRCA1* gene is located on chromosome 17q21 [126] and is composed of 22 coding exons distributed over ~100kb of genomic DNA [127]. The *BRCA1* gene encodes a large protein 1863 amino acids in length [127]. The BRCA1 protein contains an N-terminal RING motif, that interacts with BRCA1-associated RING domain protein 1 (BARD1) to form an E3 ligase, and two BRCA C terminus (BRCT) motifs that form a phosphoprotein recognition domain [128-131]. The BRCA1 protein contains a DNA-binding domain [132, 133]; and in its central region, a SQ-cluster domain (SCD), the preferred site of ATM phosphorylation that is required for efficient DNA double strand break repair [134]. It also has two nuclear localization signals (NLS) [135, 136] and a nuclear export signal (NES) [137] necessary for the predominately nuclear BRCA1 protein to shuttle between nuclear and cytoplasmic compartments [138, 139].

BRCA1 plays a role in DNA damage response, cell cycle signaling, recruitment of chromatin modifying proteins, interaction with transcription factors, and ubiquitin ligase activity;
and the loss of these functions may contribute to the development of cancer by promoting genomic instability and accumulation of cancer-causing mutations [140]. BRCA1 recognizes DNA damage and is involved in the DNA repair process via interactions with RAD51 [141, 142]. In normal cells, BRCA1 and RAD51 are essential for genomic stability predominately through the homologous recombination pathway [134] in the repair of double-strand DNA breaks. BRCA1 modulates the cell-cycle at the G2/M checkpoint [143]. In breast cancer cells, BRCA1 polyubiquitinates cyclin B and Cdc25C, two G2/M cell cycle proteins, and targets the proteins for degradation by the ubiquitin-proteasome pathway in an APC/C-independent manner [144]. BRCA1 is shown to maintain heterochromatin structure via ubiquitination of core histone H2A [145]. Reports have shown that BRCA1 physically interacts with histone acetyltransferases (HATs), p300 and CREB-binding protein (CBP) [146, 147]; and, these interactions work to enhance transcriptional activity of BRCA1 [148]. BRCA1 also functions as either a co-activator or co-repressor of transcription through its interactions with c-Myc [149], activating transcription factor 1 [150], and signal transducers and activators of transcription 1 [151] modulating their activity. BRCA1 has also been found to bind the p65/RelA subunit of the nuclear factor-κB (NF-κB) and stimulate the tumor necrosis factor-α (TNF-α) and the IL-1β-induced transcription of NF-κB target genes; suggesting BRCA1 plays a role in cell survival and cell death following cell stress [152].

Mouse models have been used to study the tumor suppressor functions of BRCA1. Deficiency in BRCA1 gene expression results in early embryonic lethality [153-155]; however, mouse models carrying allelic mutations of BRCA1 to overcome embryonic lethality resulted in cells with genomic instability and defects in DNA damage repair [154]. Still, the loss of BRCA1 alone did not lead to tumorigenesis; there was a necessity for multiple genetic alterations,
including the inactivation of p53 and the activation of oncogenes caused by genomic instability for tumorigenesis [154]. Ongusaha, et. al. showed BRCA1 plays a role in p53-directed growth suppression when both are coexpressed resulting in an irreversible senescence-like phenotype in vitro [156]. Shakya, et. al. studied BRCA1-mediated tumor suppression in genetically engineered mouse models and found the BRCT phosphoprotein recognition domain to be critical for BRCA1-mediated tumor suppression [157]. A RING mutation (C61G) disrupts the BRCA1/BARD1 heterodimer and abrogates ubiquitin ligase activity [131, 158-160]. Mice homozygous for the C61G mutation with a mutated p53 background were found to more rapidly develop tumors than in mice carrying a BRCA1-null allele [161].

There are several types of risk-associated mutations, including missense mutations, truncating mutations, and larger genomic alterations. Reported risk-associated missense mutations primarily occur in the RING and BRCT domains which are both known to be involved in functionally important protein-protein interactions [162]. The more frequently reported truncating mutations occur due to small insertion and/or deletion events that result from nonsense mutations and frameshifts [162]. Larger genomic alterations are rearrangements that result in duplications or deletions of one or more exons that can often produce a premature stop codon and may represent 7-40% of all BRCA1 mutations identified [162]. Hereditary mutation carriers have one germline mutation and later in life develop a secondary somatic mutation in the tumor through the loss of heterozygosity [163]. The loss of heterozygosity and p53 inactivation seem to be early events in the induction of hereditary OC [164].

In the study of hereditary OC and inherited BRCA1 mutations, founder mutations are very important. Founder mutations are mutations with a high frequency in individual alleles that are particular to a specific population [162]. These mutations occur when small groups of
isolated people undergo a dramatic population decrease with subsequent interbreeding and consequent reduced genetic variability; that results in a normally rare mutation continuing to be present and becoming more common within the population [165]. A well-known example of the founder effect are the Ashkenazi Jews with ancestors from Eastern and Central Europe, including Germany, Poland, Lithuania, Ukraine, and Russia [165]. Three of the best characterized BRCA1 and BRCA2 founder mutations are found in the Ashkenazi Jewish population: BRCA1 185delAG, BRCA1 5382insC, and BRCA2 6174delT [162, 166]. These three mutations represent the majority of germline BRCA1 and BRCA2 mutations found in the Ashkenazi Jewish population [167, 168]. These mutations are seen in 2.6% of the Ashkenazi Jewish population in comparison to 0.2% of BRCA1/BRCA2 mutation carriers in the general population [169, 170]; however, they contribute to 24-62% of Ashkenazi Jewish OC cases [166, 171, 172].

BRCA-associated hereditary OC is associated with favorable patient outcome in comparison to sporadic OC due to enhanced initial chemosensitivity that is lost at disease recurrence [173-176]. Also, enhanced chemosensitivity has been seen in OC with low BRCA1 expression [177, 178]. Promoter methylation status has been implicated to play a role in the enhanced chemosensitivity; however, this is still a controversial assertion. BRCA1 promoter methylation has been associated with enhanced sensitivity to platinum-based chemotherapy in \textit{in vitro} and xenograft models [179]. Another study found increased survival time in patients with methylated BRCA1 promoter and fewer cases of recurrent epithelial OC in patients with methylated BRCA1 promoter suggesting that BRCA1 promoter methylation is predictive for response to platinum-taxane-based chemotherapy [180]. In opposition, investigation of 316 OC patients found neither BRCA1 mutation status nor its promoter methylation has been associated
with patient outcome [181]. Two other clinical trials likewise found no correlation between BRCA1 promoter methylation and chemosensitivity [182, 183].

BRCA1 mutations can result in complete or partial loss of wild-type function. However, there are BRCA1 mutations that result in protein products that may have functions independent of wild-type BRCA1 or in competition to wild-type BRCA1 function. For instance, the 5677InsA insertion mutation, which results in a frameshift and premature stop signal at codon 1853, inhibits proliferation of human prostate cancer cells more efficiently than endogenous wild-type BRCA1 [184]. The ability of wild-type BRCA1 to enhance apoptosis in breast and OC cells was inhibited when co-expressed with BRCA1 mutations, 5382InsC and 5677InsA [185]. The BRCA1 mutant, 185delAG, results in a 66% lifetime risk of developing OC [186]. The BRCA1 185delAG Amino Terminal truncated protein or BRAT is a result of a deletion of two nucleotides (AG) in the second exon of the BRCA1 gene leading to a reading frame shift and a premature stop codon at exon 39; as well as, complete loss of the BRCT motifs. BRCA1 gene mutations resulting in a premature stop codon are generally susceptible to nonsense-mediated messenger RNA (mRNA) decay. However, 185delAG and 5382InsC, were found to be unaffected by mRNA decay [187, 188].

In order to improve survival rates, it is necessary to determine the elements involved in premalignant changes leading to early stage oncogenesis in the possibility of determining additional cancer markers for early stage diagnosis. The greatest confounder to understanding premalignant changes leading to early stage disease is an unknown etiology. One obstacle to understanding OC etiology is the lack of an appropriate experimental mouse model. While researchers have been able to induce ovarian-like tumor models in certain genetically-altered mice or genetically-altered cell lines injected into mice, the reoccurring limitation is an inability
for the disease to be inherited by the progeny leading to spontaneous OC formation (reviewed in [189]). The spontaneous formation of heritable OC is required to observe the changes from premalignancy to malignancy.

**Rationale**

Previously, we demonstrated that OSE from carriers of the BRCA1 185delAG mutation exhibited enhanced apoptosis and caspase 3 activation in response to staurosporine [190]. In order to rule out the possibility that these features were due to the loss of bi-allelic BRCA1 gene, BRAT was expressed in OSE cells with endogenous wild-type bi-allelic BRCA1 gene. In agreement, BRAT cells also exhibited enhanced caspase 3-mediated apoptosis; as well as, diminished levels of phospho-Akt, cellular inhibitor of apoptosis 1 (cIAP1), and X-linked inhibitor of apoptosis protein (XIAP) [191]. Further, we found that BRAT upregulated expression of the tumor suppressor maspin and correlated with enhanced chemosensitivity [192]. By contributing to apoptosis, invasion and metastasis, maspin has been correlated with cisplatin sensitivity in OC cell lines and increased overall survival times in OC patients [193]. Lastly, we have also shown that matrix metalloproteinase-1 (MMP1) is transcriptionally upregulated in a c-Jun-dependent manner and that BRAT OSE cells or BRCA1 185delAG OSE cells secrete enhanced levels of MMP1 (Linger, publication in progress).

These studies taken together demonstrate that the BRCA1 185delAG mutation mediates unique molecular and cellular changes in OSE cells independent of the loss of endogenous BRCA1 function. These changes could impact the physiology of mutation carriers. Therefore, it is important to identify additional BRAT targets and cellular processes important in OC initiation and progression. While family history, usually associated with BRCA1 mutations, remains the
greatest risk factor for OC, inflammation may play an important secondary role in development of disease. Preliminary microarray analysis (Table 1.2) showed increased IL-1β gene expression in human immortalized OSE transiently expressing exogenous BRAT (Johnson, Kruk, unpublished data) suggesting IL-1β as yet another target of BRAT. Therefore, the risk for OC by BRCA1 185delAG may be mediated, in part, by the ability of 185delAG/BRAT to promote an inflammatory phenotype.

Central Hypothesis

I hypothesize that expression of the 185delAG BRCA1 mutant protein product, BRAT, alters the regulation and expression of the proinflammatory cytokine, IL-1β; which, in turn, may be of clinical relevance.

Specific Aims

To address this central hypothesis, I propose three specific aims.

1. Confirm IL-1β as a downstream target of 185delAG in immortalized human OSE cells.
2. Determine the clinical correlation of urinary IL-1β among gynecologic patients.
3. Establish the tools to determine the mechanism by which BRAT mediates novel gain of function.
Table 1.2 Microarray Condensed Results Showing Gene Expression Change.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Change PCDNA3.1 vs BRAT</th>
<th>Average fold change</th>
</tr>
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<tbody>
<tr>
<td>IL1α</td>
<td>I</td>
<td>4.61</td>
</tr>
<tr>
<td>IL1β</td>
<td>I</td>
<td>3.84</td>
</tr>
<tr>
<td>IL6</td>
<td>I</td>
<td>2.50</td>
</tr>
<tr>
<td>MMP1</td>
<td>I</td>
<td>2.70</td>
</tr>
</tbody>
</table>

References


Chapter 2:

BRCA1 185delAG Mutation Enhances Interleukin-1β Expression in Ovarian Surface Epithelial Cells

Introduction

Ovarian cancer (OC), an inflammation associated cancer, is the deadliest gynecologic malignancy and is the 9th most common cancer among women [1]. The American Cancer Society estimates that approximately 22,240 new cases will be diagnosed and 14,030 women die of the disease annually, respectively [1]. The 5-year survival rate is 92% when diagnosed in early stage and yet confined to the ovary [1]. However, only about 15% of cases are diagnosed in the early stage and the 5-year survival rate drops to approximately 27% when diagnosed in later stages [1].

Most OCs are epithelial ovarian carcinomas traditionally thought to arise from the ovarian surface epithelium (OSE) [2]; though more recently, studies have suggested that OC may arise from the fallopian tube epithelium [3, 4]. The etiology of the disease is not completely understood, but family history (FH) is the strongest risk factor for the development of epithelial OC [5]. Hereditary OCs are often associated with mutation of the tumor suppressor breast cancer susceptibility gene 1 (BRCA1) [6]. Carriers of the BRCA1 mutation have a 30% risk of developing OC during their lifetime [7]. BRCA1 plays a role in DNA damage response, cell cycle signaling, recruitment of chromatin modifying proteins, interaction with transcription
factors, and ubiquitin ligase activity [8]. Loss of these functions may contribute to the development of cancer by promoting genomic instability and accumulation of cancer-causing mutations. Mutation of the BRCA1 gene can result in either “loss of function” or “gain of function” with appearance of novel truncated protein products, respectively (reviewed in [9]). Among possible gain of function BRCA1 mutations, the 185delAG mutation is one of the most common founder mutations and is associated with a 66% lifetime risk of developing OC [10].

The 185delAG BRCA1 truncated mutant, BRAT, is a result of a deletion of two nucleotides in the second exon of the BRCA1 gene leading to a reading frame shift and a premature stop codon at exon 39. Previously, we have demonstrated that human OSE cells with the BRAT mutation exhibited enhanced apoptosis and caspase-3 activation in response to staurosporine [11] as well as diminished levels of phosphorylated Akt, cellular inhibitor of apoptosis 1 (cIAP1), and X-linked inhibitor of apoptosis protein (XIAP) [12]. We have also found that BRAT up-regulated maspin expression was correlated with enhanced chemosensitivity [13] which is in agreement with clinical reports of increased survival in patients with elevated maspin levels [14]. Microscopic examination of ovarian specimens obtained following prophylactic oophorectomy from women with family history (FH) of OC indicated that greater than 85% presented with two or more abnormal OSE histologic features such as surface epithelial pseudostratification, surface papillomatosis, cortical invaginations of OSE epithelial inclusion cysts and epithelial hyperplasia [15]. Likewise, overtly normal FHOSE in culture show an increased autonomy of the epithelial phenotype in terms of expression of the epithelial differentiation marker CA125 [16], persistence of an epithelial morphology [16], and a reduction in epithelial-mesenchymal conversion as noted by the maintenance of high keratin expression, but reduction of collagen type II expression compared with no family history (NFH)
OSE [17]. Since OSE become more firmly committed to an epithelial phenotypic in the course of carcinogenesis, these reports coupled with our previous findings suggest that pre-neoplastic characteristics may already exist in overtly normal OSE in some women with a strong family history of breast and OC.

Inflammation of the ovarian epithelium has long been associated with increased risk for OC [18-21]. By promoting a local pelvic inflammatory reaction, endometriosis has been associated with increased risk for endometrioid adenocarcinoma and clear cell carcinoma of the ovary [22-25]. Likewise, chronic pelvic inflammatory disease, often resulting from infection, also supports a role for inflammation and increased risk for OC [26, 27]. Lastly, epidemiological studies suggest that incessant ovulation causes rapid cycles of OSE division associated with repeated ovulatory traumatization and repair of the ovulatory defect [28]. Ultrastructural and histochemical studies of OSE in situ have shown that OSE migrates and proliferates extensively during repair of the OSE after ovulation [29, 30]. During the ovulatory repair, the OSE is exposed to proinflammatory mediators such as, cytokines, chemokines, matrix-remodeling enzymes, and various growth factors that can result in an increased risk of malignant transformation ([31, 32]). Reports of OC associated with ovarian hyperstimulation due to fertility drugs [33, 34] further support a role for inflammatory traumatization in OC.

Interleukin-1β (IL-1β) is a proinflammatory cytokine mainly produced by monocytes and macrophages [35], but can also be produced by endothelial cells, fibroblasts, and epidermal cells in response to bacterial or innate immunity stimulation [36]. Interestingly, normal and malignant epithelial ovarian cells also produce IL-1β [2]. IL-1β is translated into a 31kDa inactive precursor form that is cleaved intracellularly by caspase-1 into an active 17kDa secreted form [35]. The aim of this study was to investigate the in vitro production of IL-1β in human OSE.
cell lines carrying the 185delAG BRCA1 mutation in order to determine whether enhanced IL-1β in these cells could contribute to an inflammatory phenotype.

**Materials and Methods**

**Cell Culture and Transfection**

The following SV 40-Large T-Antigen transfected human OSE (IOSE) cell lines were used: IOSE118 derived from a normal patient with a family history (FH) of breast and/or OC with wtBRCA1 status confirmed [13]; IOSE 121 derived from a normal patient with no family history (NFH) of breast and/or OC though BRCA1 mutation status was not determined and; IOSE 3261-77 and IOSE 1816-686 derived from normal patients with a FH of breast and/or OC as well as confirmed carriers of the BRCA1 185delAG mutation. IOSE cells were cultured in Medium 199/MCDB 105 (Sigma, St. Louis, MO) with 10% fetal bovine serum (FBS) and gentamicin. Multiple stable BRAT clones were generated by transfection of 2.5 x 10^5 IOSE 118 cells with 2.5ug of PCDNA3.1 or Flag-BRAT with G418 resistance gene as previously described [13] using Program X-005, Kit V, and the Nucleofector device (Amaxa/ Lonza, Walkersville, MD). BRAT clones were designated BRATc1, BRATc2, BRATc3, and BRATc4. Stable cells were maintained in 1mg/ml G418 selection media and confirmed to express BRAT by RT-PCR [13]. All cells were incubated at 37°C with 5% CO₂. For knockdown studies, cells were co-transfected with 1.5ug ON-Targetplus siRNA (SiCreb, SiCon) from Dharmacon (Chicago, IL). For treatment with IL-1 Receptor antagonist (IL-1Ra), stable BRAT clones were washed with phosphate buffered saline (PBS) and media containing 0.1% FBS was added to the cells along with varying concentrations of IL-1Ra (R&D Systems, Inc., Minneapolis, MN). After 6 hours,
the media was removed and RNA was extracted from the cells for PCR analysis as described below.

MCF7, SkBr3, and MDA-MB-231 human breast cancer cells were cultured in Medium 199/MCDB 105 with 10% FBS and gentamicin. MCF10A normal human breast epithelial cells were cultured in DMEM/F12 (Mediatech, Manassas, VA) supplemented with 15mM HEPES, insulin 10ug/mL, EGF 20ng/mL (Sigma, St. Louis, MO), cholera toxin 100ng/mL (Biomol, Plymouth Meeting, PA), hydrocortisone 0.5ug/mL (BD Biosciences, Sparks, MD), L-glutamine (MP Biomedicals, Solon, OH), glucose, sodium bicarbonate, 10% FBS, and gentamicin. All cells were incubated at 37°C with 5% CO₂. Two million cells were transiently transfected with BRAT as previously described [13] using Program X-005 (for MCF7 cells), E-09 (for SkBr3 cells), X-013 (for MDA-MB-231 cells), T-024 (for MCF10A cells), Kit V, and the Nucleofector device (Amaxa/ Lonza, Walkersville, MD) with 2.5-3.5ug of plasmid (pcDNA3.1 or Flag-BRAT).

**Western blot**

Cells were washed in PBS, trypsinized, pelleted, and washed 1-2 times in cold PBS. Cells were lysed for 30 minutes on ice in modified CHAPS buffer, and lysate was centrifuged at 115,000 x g, at 4°C for 1h. Then 30ug of protein were separated via 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to Polyvinylidene fluoride (PVDF) membranes, dried, and blocked in 5% milk in Tween 20-Tris buffered Saline. Blots were incubated in their respective antibodies overnight, followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary (Fisher, Pittsburgh, PA), and developed via enhanced chemiluminescence substrate (ECL) (Pierce/ Fisher, Pittsburgh, PA). Antibodies: IL-1β (1:1000) Cat. # 2022 Cell Signaling Technology (Beverly, MA), Actin clone AC-40
(1:10,000) Cat. # 4700 Sigma (St. Louis, MO), Apoptosis-associated Speck-like protein containing a C-terminal caspase recruitment domain (ASC) (1:1000) Cat. # sc-271054 Santa Cruz Biotechnology, Inc. (Dallas, TX), Caspase-1(1:1000) Cat. # 2225 Cell Signaling Technology (Beverly, MA), CREB (1:1000) Cat. # 9104 Cell Signaling Technology (Beverly, MA), NACHT, LRR and PYD domains-containing protein 3 (NALP3) (1:1000) Cat. # sc-134306 Santa Cruz Biotechnology, Inc. (Dallas, TX). Western blot quantification was done in ImageJ software normalizing band strength to the actin band.

**Enzyme-linked Immunosorbent Assay**

For conditioned media analysis, media containing 0.1% FBS was added to cells 24 hours after transfection/plating. After 24 hours, cells were counted and media was collected and centrifuged to remove debris, aliquotted, and stored at (-80°C). For the IL-1β Enzyme Linked Immunosorbent Assay (ELISA), conditioned media was also concentrated 17x using Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-10 membrane (Millipore, Billerica, Massachusetts) before storage at (-80°C). To assess the presence of mature IL-1β, an ELISA (R&D Systems, Inc., Minneapolis, MN) was performed on concentrated conditioned media samples in triplicate according to manufacturer’s protocol. Fluorescence was read on an ELx800 Absorbance Microplate Reader (Biotek, Winooski, Vermont) using Gen5 Data Analysis Software (Biotek, Winooski, Vermont). Resultant values were derived from a standard curve and expressed as the mean IL-1β concentration of triplicate samples ± standard error. When cell viability varied significantly, IL-1β concentration was normalized to average cell number at time of conditioned media collection.
PCR

RNA samples were isolated using TRIzol reagent from Invitrogen (Carlsbad, CA) per manufacturer’s protocol and DNAse treated.

For BRAT semi-quantitative PCR to confirm transfection, 1ug total RNA, oligo(dT), and reverse transcriptase were used to generate single-strand cDNA as previously described [13]. The cDNA samples were amplified using the Applied Biosystems GeneAmp RNA PCR Core Kit (Foster City, CA). Primers used were: Flag-BRAT sense (CGATGACAAAATGGATTTATCTGC), Flag-BRAT antisense (GAGACAGGTTCCCTCATCAACTCC), actin sense (GGGAATTCAAAACTGGAACGGTGAAGG), and actin antisense (GGAAGCTTATCAAAGTCTCGGCCACA). The amplified products were separated by electrophoresis on a 10% polyacrylamide gel, stained with SYBR Green (Lonza, Rockland, ME), and photographed with the Kodak EDS 120 Digital Analysis System. The net intensity of each band was normalized to the respective endogenous control band.

For quantitative PCR, 100ng total RNA was reverse transcribed to generate single-strand cDNA as previously described [13]. The cDNA samples were amplified in triplicate using Fast SYBR Green Master Mix (Applied Biosystems) on an Applied Biosystems Step One Plus instrument. Primers used were: IL-1β sense (TCCAGGGACAGGATATGGAG), IL-1β antisense (TCTTTCAACACGCAGGACAG), and actin (same as above). RQ (relative mean mRNA expression level) was calculated by the Step One software version 2.0. Using standard curves constructed for target and endogenous control genes, an arbitrary quantitative gene expression value was determined from the threshold cycle (Ct) for each gene for each sample.
Target gene values were normalized to control gene values, and fold difference was determined by dividing by the designated reference/calibrator sample.

**Dual-Luciferase assay**

Stable 118 PCDNA3.1 or BRAT clones were transfected with 0.15μg Renilla luciferase reporter and 1.5μg IL-1β luciferase promoter deletion constructs pIL1(4.0kb)LUC (-4000), pIL1(3.1kb)LUC (-3100), pIL1(1.8kb)LUC (-1800), pIL1(0.9kb)LUC (-900), and pIL1(0.5kb)LUC (-500). Twenty-four hours later, cells were collected in Promega Passive Lysis Buffer and subjected to two freeze-thaw cycles. Lysates were centrifuged at 10,600 x g for 1 minute at 4°C, and supernatant was collected. Luciferase activity was assessed in triplicate using a manual luminometer and the Promega Dual Luciferase Assay System according to manufacturer protocol. For knockdown reporter studies, siRNA was co-transfected and cells were collected 48 hours after transfection.

**Immunoprecipitation**

Cells from stable 118 PCDNA3.1 and BRAT clones were washed with PBS incubated on ice with RIPA buffer containing protease inhibitors. After 15 minutes, the cells were removed by scraper and lysate was incubated for 60 minutes on ice. The cell suspension was centrifuged at 10,000 x g for 10 minutes at 4°C. Two micrograms of primary antibody was incubated with 1mg of whole cell lysate for 2 hours at 4°C. Protein A/G agarose suspension was added to lysate and antibody mixture followed by incubation at 4°C on a rocker overnight. The suspension was collected by centrifugation at 500 x g for 2 minutes at 4°C. The cell pellet was washed with RIPA buffer containing protease inhibitors followed by analysis by western blot.
Statistics

For real time PCR, error bars illustrate RQmin and RQmax, which are calculated as: RQave divided by (standard deviation^ student’s t value at the 95% confidence interval, for 5 degrees freedom) and RQave times (standard deviation^ student’s t value at the 95% confidence interval, for 5 degrees of freedom), respectively. This range represents the confidence interval at the 95% confidence level. For ELISA and reporter assay data, student’s t test was performed to assess statistical difference between means of triplicates ± standard error from three separate experiments.

Results

**IL-1β levels are increased in IOSE cells carrying BRCA1 185delAG mutation.**

To determine the relationship between the BRCA1 185delAG mutation and the protein levels of IL-1β, I compared IL-1β protein levels in human OSE cell lines that endogenously carry this mutation to those with wild-type BRCA1 mutation. Both FH IOSE cell lines (3261-77 and 1816-686) with confirmed 185delAG mutation had at least 7-fold higher pro-IL-1β protein levels as well as ≥ 13-fold higher active IL-1β protein levels as measured by western blot than the NFH cell line (IOSE-121) (Figure 2.1A). I further examined secreted IL-1β protein levels in these same cell lines. The 3261-77 and 1816-686 cell lines had 2- and 10-fold, respectively, higher levels of secreted IL-1β protein in their conditioned media as measured by ELISA than the IOSE-121 cell line (Figure 2.1B).

**BRAT transfection increases levels of IL-1β in IOSE cells.**

To confirm that the increased intracellular and secreted IL-1β levels are related to the
Figure 2.1. **IL-1β protein levels are increased in 185delAG BRCA1 mutation carriers.** (A) Normal IOSE cells were analyzed for precursor (pro-) and cleaved IL-1β protein expression via western blot. Blots were then stripped and probed for β-actin as a loading control. Values represent relative densitometry. (B) Cells were plated in triplicate at similar densities and conditioned media was collected as described. IL-1β ELISA activity assay was performed in triplicate as described. Graph illustrates mean ± standard error. Symbol (*) denotes statistical significance at the 0.04 confidence level.
presence of BRAT, I analyzed IOSE-118 cells stably transfected with PCDNA3.1 or Flag-BRAT for IL-1β protein levels. BRATc1 had 2.7-fold higher levels of intracellular IL-1β protein as measured by western blot than the PCDNA3.1 cell line (Figure 2.2A). Furthermore, BRATc1 and BRATc2 both had 2-fold and 1.5-fold, respectively, higher levels of secreted IL-1β protein levels as measured by ELISA than the PCDNA3.1 cell line (Figure 2.2B). To determine whether BRAT-mediated IL-1β expression is transcriptionally regulated, I evaluated IL1β mRNA level in BRAT-expressing IOSE-118 cells. Stably transfected IOSE-118 BRAT cells showed up to a 3-fold increase in IL1β mRNA expression as measured by real time PCR, compared to PCDNA3.1 cells (Figure 2.2C).

**BRAT-mediated IL1β expression is tissue specific.**

To determine whether BRAT-mediated enhanced IL1β mRNA levels is specific to ovarian epithelial cells, normal breast epithelial (MCF-10A) and breast cancer (MDA-MB-231, SK-BR-3, and MCF-7) cell lines were transiently transfected with PCDNA3.1 or BRAT transcript. Real time PCR of the breast cell lines showed no change in IL1β mRNA in the PCDNA3.1 and the BRAT transfected cell lines (Figure 2.3).

**BRAT-mediated IL1β promoter activity is partially mediated by CREB in IOSE cells.**

To determine which region of the IL1β promoter was required for the BRAT-mediated IL1β promoter activation, I utilized dual-reporter assays in BRAT cells using five truncated IL1β promoter luciferase reporter plasmids. There was no difference in reporter activity between deletion constructs (-4000), (-3100), and (-1800) (Figure 2.4A). However, reporter activity was diminished by 80% between deletion constructs (-1800) and (-900) (Figure 2.4A). Furthermore, there was no difference in reporter activity between deletion constructs (-900) and (-500) (Figure 4A).
Figure 2.2. IL-1β protein and message levels are increased in stably transfected 185delAG OSE cells. (A) IOSE 118 cells were transfected with pcDNA3.1 (control) or 185delAG transcript (BRATc1) and subsequently analyzed for precursor (pro-) and cleaved IL-1β protein expression via western blot. Blots were then stripped and probed for β-actin as a loading control. Values represent relative densitometry. (B) pcDNA3.1, BRATc1, and BRATc2 cells were plated in triplicate at similar densities and conditioned media was collected as described. IL-1β ELISA activity assay was performed in triplicate as described. Graph illustrates mean ± standard error. Symbol (*) denotes statistical significance at the 0.01 confidence level between BRAT and PCDNA3.1 cells. (C). Cells stably expressing BRAT or the pcDNA3.1 cells were plated at equal densities and collected. RNA was isolated, DNAse treated, and reverse transcribed. Real-time PCR was performed in triplicate for IL-1β and actin using SYBR green detection. Graph illustrates mean ± standard error.
Figure 2.3. BRAT-mediated IL-1β expression is tissue specific. MCF-10A (normal breast epithelial), SK-BR-3 (breast cancer), and MCF-7 (breast cancer) cells were plated at equal densities, transiently transfected with either BRAT or the pcDNA3.1 vector, and RNA was collected 48 hours after transfection. RNA was isolated, DNAse treated, and reverse transcribed. Real-time PCR was performed in triplicate for IL-1β and actin using SYBR green detection. Graph illustrates mean ± standard error.
Figure 2.4. CREB sites within the IL-1β promoter mediate enhanced IL-1β mRNA expression in BRAT cells.  (A) Cells stably expressing BRAT were transiently transfected with the indicated IL-1β deletion reporter construct and a Renilla constitutive luciferase reporter plasmid for normalization.  Lysates were collected, subjected to two freeze-thaw cycles, and assayed in triplicate on a manual luminometer using Promega’s Dual Luciferase Assay kit.  Luciferase activity was normalized to Renilla luciferase activity for each triplicate, averaged, and results are expressed as mean ± standard error.  (B) Cells stably expressing BRAT were also co-transfected with non-targeting control siRNA (siCon) or siRNA targeting CREB, collected, and assayed similarly.  Protein lysates were collected in parallel for knockdown analysis and CREB protein silencing demonstrated by western blot (inset).  Symbol (*) denotes statistical significance ≤ 0.03 confidence level.
This suggests that the 900bp region between the (-1800) construct and the (-900) construct contributes significantly to BRAT-mediated \( \text{IL1} \beta \) promoter activity.

To further evaluate BRAT-mediated \( \text{IL1} \beta \) promoter activation, I performed an online transcription factor prediction analysis of the 900bp promoter region identified earlier to determine potential transcription factor binding sites using the prediction software, PROMO [37, 38]. My analysis identified two potential CREB binding sites within the 900bp promoter region. Based on this observation, I used pooled siRNA to knockdown CREB and western blot to confirm CREB silencing. I then analyzed \( \text{IL1} \beta \) promoter activity using the (-1800) reporter plasmid. Knockdown of CREB resulted in partial loss of reporter activity (Figure 2.4B). This data suggests that CREB is at least partially required for activation of the \( \text{IL1} \beta \) promoter in BRAT cells.

**Caspase-1 protein expression is increased in BRAT IOSE cells.**

To confirm the intracellular processing of precursor inactive IL-1\( \beta \) into its active form, I measured caspase-1 levels, the known intracellular activator of IL-1\( \beta \). Stably transfected PCDNA3.1 cells had about 2-fold more inactive precursor caspase-1 protein levels than BRAT cells as seen by western blot (Figure 2.5). However, stably transfected BRAT cells showed up to a 50-fold increase in active caspase-1 as seen by western blot (Figure 2.5).

**ASC protein expression is increased in BRAT IOSE cells.**

BRAT increases precursor IL-1\( \beta \) protein by increased transcription and concurrently increases active caspase-1 by unknown mechanisms to accommodate cleavage of precursor IL-1\( \beta \) to active IL-1\( \beta \). To determine the control of enhanced activation of caspase-1 by the NALP3-ASC inflammasome in BRAT cells, the presence of NALP3 and ASC were determined by Co-IP and visualized by western blot.
Figure 2.5. Cleaved caspase-1 is elevated in BRAT cells. Cells stably expressing pcDNA3.1 or BRAT (BRATc1 & BRATc2) were analyzed for precursor (pro-caspase-1) and cleaved caspase-1 (caspase-1 p20) protein expression via western blot. Blots were then stripped and probed for β-actin as a loading control. Values represent relative densitometry.

Stably transfected PCDNA3.1 and BRAT cells were immunoprecipitated with an ASC antibody, followed with a western blot probed for NALP3 and ASC. PCDNA3.1 and BRAT cells showed similar protein levels of NALP3; however, BRAT cells showed an increase in ASC protein levels (Figure 2.6A). Additional western immunoblots confirmed a 2.5 fold increase of ASC protein levels in BRAT cells compared to the PCDNA3.1 cells (Figure 2.6B).

**IL-1Ra suppresses proinflammatory mediators in BRAT IOSE cells.**

To evaluate the potential auto-feedback loop of IL-1β on OSE cells, I treated stably transfected BRAT-containing IOSE cells with the IL-1 receptor antagonist, IL-1Ra. Then, I measured mRNA expression levels of known IL-1β downstream transcription targets: IL-1β, IL-6, and IL-8 by real time PCR. Treatment of BRAT cells with IL-1Ra resulted in about a 50% loss of mRNA expression levels of IL-1β, IL-6, and IL-8, respectively, at the 0.5ng/mL treatment level (Figure 2.7).
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IB: NALP3
IP: ASC
IB: ASC

B.

Figure 2.6. BRAT alters levels of inflammasome constituents. (A) Cells stably expressing pcDNA3.1 or BRATc4 were immunoprecipitated with the ASC antibody, then analyzed via western blot for ASC and NALP3. Values represent densitometry. (B) Cells stably expressing pcDNA3.1 or BRAT were analyzed for ASC protein expression via western blot. Blots were then stripped and probed for β-actin as a loading control. Values represent relative densitometry.
Figure 2.7. IL-1Ra inhibits IL-1β, IL-6, and IL-8 mRNA expression in BRAT cells. Cells stably expressing BRAT were plated at equal densities, treated with recombinant IL-1Ra and collected. RNA was isolated, DNase treated, and reverse transcribed. Real-time PCR was performed in triplicate from two separate experiments for IL-1β, IL-6, IL-8, and actin using SYBR green detection. Graph illustrates mean ± standard error.

Discussion

The results of the present study show that the 185delAG BRCA1 mutant protein, BRAT, increases IL-1β mRNA and protein levels in immortalized human OSE cell lines. Furthermore, I have demonstrated that BRAT-dependent expression is transcriptionally mediated, in part, via CREB binding sites. I have also demonstrated that BRAT-dependent activation of IL-1β protein...
by caspase-1 may be enhanced by increased ASC protein expression of the NALP3-ASC inflammasome. While further investigation is required to discover the exact mechanism of enhanced ASC protein expression in BRAT cells, I suspect ASC expression may be CREB-dependent or AP1-dependent since the current and prior studies indicate BRAT-mediated gene expression through CREB and AP1 sites [39] and CREB binding sites are potentially present in the ASC promoter according to commercially available promoter ChIP assay (Qiagen, Valencia, CA) program analysis. Lastly, I have shown that IL-1β plays a role in the induced expression of proinflammatory mediators, IL-6 and IL-8 via the IL-1 receptor suggesting that BRAT might stimulate a proinflammatory environment that could promote OC oncogenesis.

Establishing animal and cell models of OC oncogenesis has proven to be difficult. Spontaneous development of OC in animal models occurs with a low frequency and highly variable phenotype [40]. Furthermore, OC lacks a clear molecular profile making it difficult to elucidate a universal cause in OC initiation or progression [41-43]. Loss of some or all wild type functions of the BRCA1 gene product due to gene mutation is commonly associated with enhanced breast cancer and OC risk (Reviewed in [9]). BRCA1 gene mutations resulting in a premature stop codon are generally susceptible to nonsense-mediated messenger RNA (mRNA) decay. However, two common risk-associated BRCA1 mutations, 185delAG and 5382InsC, were found to be unaffected by mRNA decay [44, 45]. This study utilizes a stable OSE cell line model with intact endogenous bi-allele BRCA1 along with the 185delAG BRCA1 mutant transcript. By retaining intact BRCA1, I am able to conclude the changes observed are due to the presence of the 185delAG BRCA1 mutation and not due to the partial or complete loss of BRCA1. This is in keeping with previous studies that have shown other BRCA1 mutations with
independent and novel gain-of-function roles in OC proliferation, chemosensitivity, tumorigenesis, and apoptosis [46, 47].

Mutation of the \textit{BRCA1} gene is associated with the majority of hereditary OCs resulting in a 30\% lifetime risk of developing OC [7]. \textit{BRCA1} gene mutations are also associated with 50-80\% risk of developing breast cancer before 70 years of age [48]. The propensity for gender- and tissue-specific \textit{BRCA1}+ disease has long been thought to be associated with estrogen in these hormone-responsive tissues [49] such that aberrations in the regulation and signaling interactions between \textit{BRCA1} and estrogen [50-53] promote breast and/or OC. Similar tissue specific disease development associated with gene mutations is not uncommon. For instance, germline mutation of the \textit{ALK} gene is associated with familial neuroblastoma, but can also be associated with lymphoma and lung cancer depending on the genetic aberration that occurs [54]. However, not all \textit{BRCA1} germline carriers develop disease and carriers of a mutation rarely develop both ovarian and breast cancer. A recent study by Maxwell et al. indicated that combined mutations in RHAMM secondary to \textit{BRCA1} mutations promoted Aurora kinase A- and TPX2-mediated disorganization of epithelial polarity, thereby potentiating breast carcinogenesis [55]. Similarly, I may have not noted changes in \textit{IL1β} expression in breast tissue with the 185delAG plasmid because of the necessity of further genetic alterations needed to initiate breast cancer transformation. Interestingly, the IOSE cells employed in this study overexpress p53 [56] as a result of nuclear sequestration by the large-T antigen which interferes with the growth regulatory activities of p53 [57] and which might mimic the high-grade, ‘type II’ pathway for OC development arising from p53 mutations [58]. However, given the range of p53 status from wild-type to mutated/overexpressed to mutated/deficient among the breast cancer cell
Inflammation has long been suggested to contribute to tumor initiation, promotion, and progression [63]. Many components of the inflammatory pathway, including free radicals, cytokines, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and vascular endothelial growth factor (VEGF) have been implicated in the development of various malignancies, including OC [64]. Immunohistochemical analyses revealed increased COX-2 expression in non-mucinous ovarian tumors and with increased COX-2 expression correlating with poor prognosis [65]. Likewise, the present results are in keeping with others who reported a relation between proinflammatory mediators and increased OC risk. A case-control study confirmed a positive association between circulatory inflammatory cytokines, IL-2, IL-4, IL-6, IL-12, and IL-13, and OC risk [66]. Also, increased serum levels of inflammatory marker CRP were associated with an increased risk of OC [20, 67]. Yellapa et al., demonstrated increased expression of the proinflammatory cytokine, IL-16, in serum and tissue of early and late stage OC patients; as well as, increasing IL-16 serum levels during the progression from normal to early and late stage OC progression in hens [68]. Further, since increasing IL-16 serum levels preceded detection of OC by transvaginal ultrasound, it is tempting to speculate that monitoring proinflammatory mediators, such as IL-1β, may signal disease in women at high risk for OC before detection by conventional means.

In contrast, epidemiological studies suggest that long-term usage of non-steroidal anti-inflammatory drugs (NSAIDs) is associated with decreased OC risk [69-71]. Further, *in vitro* and *in vivo* use of phytochemicals [72] exert anti-inflammatory activity related to reduced OC progression by stabilizing p53 [73], enhancing cisplatin sensitivity [74], inhibiting cell growth
and VEGF expression [75] and suppressing IL-6 function [76]. There are currently three IL-1 inhibitors used in the treatment of non-malignant inflammatory conditions: recombinant IL-1Ra, anakinra; IL-1 trap, rilonacept; and monoclonal anti-IL-1β antibody, canakinumab [77]. Consequently, premalignant targeting of the proinflammatory mediator IL-1β and its upstream processors, caspase-1 and/or the NALP3 inflammasome, specifically in 185delAG BRCA1 mutation carriers, may reduce the risk of malignant transformation initiated by chronic inflammation.

Lastly, tumors can develop within and in response to their inflammatory microenvironment. For example, Kim et al., observed localized IL-1 expression among normal, benign, and malignant canine mammary tumors such that IL-1 could not be detected in normal tissue while concentrated IL-1 expression was noted in the stroma of benign tumors and diffused expression in malignant and metastatic tumors [78]. Similarly, IL-1β secreted by OC cells suppresses p53 expression in cancer-associated fibroblasts [79]. In OC, IL-1β also promotes invasiveness, tumor angiogenesis, and induces immune suppression [80]. Constitutive production of IL-1β by human ovarian carcinoma cell lines [81] enhances their invasion capacities by increasing expression of matrix metalloproteinase-1 and stimulating production of pro-angiogenic factors [82, 83]. Watanabe, et al. showed that IL-1β produced by OC cells induced mesothelial cell beta1-integrin dependent peritoneal metastasis [84]. Increased levels of IL-1β found in the serum and/or the ascites of OC patients was associated with decreased survival [85]. Likewise, IL-1β can affect OC progression by altering the expression of other proinflammatory cytokines, such as IL-6 which is a downstream target of IL-1β. IL-6 is known to play a major role in OC progression and prognosis; and may also be a potential marker of immunological and metabolic changes in OC [86]. Elevated levels of IL-6 was found in the
serum and ascites of OC patients [87]; and was also associated with poor prognosis [2]. Therefore, the chronic presence of IL-1β within the ovarian microenvironment may enhance malignant transformation and underscores the complexity among cancer cells within their microenvironment for tumor progression.

The data identify the proinflammatory mediator, IL-1β, as a novel target in OSE cells expressing the 185delAG BRCA1 mutation. Further, the mechanism by which BRAT regulates IL-1β expression is twofold: (1) CREB-dependent transcriptional control and (2) caspase-1 protein cleavage of IL-1β. Further studies on the role of BRCA1 mutations to promote a chronic inflammatory phenotype are warranted since the potential exists for IL-1β to serve as a predictor of OC and/or as a therapeutic target.

References


Chapter 3:

Urinary Interleukin-1β Levels Among Gynecological Patients

Introduction

Ovarian cancer (OC) is the fifth leading cause of cancer death among women after lung, breast, colorectal, and pancreatic cancer [1]. OC is highly treatable when diagnosed in the early stages; however, the majority of cases are diagnosed in the late stage and 5-year survival is approximately 27% [1].

In order to detect early stages of OC and avoid unnecessary surgery, there is a need for a better understanding of the etiology of this disease as well as improved screening options. Currently, there are three screening procedures in use for OC detection: bimanual pelvic examination, serum CA125, and transvaginal ultrasonography (TVS) [2]. Pelvic examinations are not effective in distinguishing a premalignant lesion from a normal ovary [3]. Serum CA125 is elevated in 47% of women with early stage OC and elevated in 80-90% of advanced stage OCs [4]. However, CA125 can also be elevated in healthy women and in patients with benign ovarian disease [5]; as well as, in other cancers such as endometrial, pancreatic, lung, breast, colorectal, and certain gastrointestinal tumors [6]. The pairing of TVS with serum CA125 improves OC diagnosis; however, sonography can result in false positive results and unnecessary surgery [7]. Unfortunately, utilizing these methods either alone or in combination does not
produce the desired results for early disease detection. Therefore, examining the molecular changes that occur at early disease onset may provide new approaches or biomarkers for OC detection.

Deregulation of inflammation due to overexpression of proinflammatory proteins contributes to the malignant phenotype by supporting cancer cell growth and tumor invasion. Since the proinflammatory cytokine, interleukin-1β (IL-1β), is: constitutively expressed in OC [8] elevated in serum of OC patients [9]; expressed in cultured OC and FH IOSE cells (chapter 2) and; secreted by ovarian cells (chapter 2), I sought to assess whether elevated urinary levels of IL-1β are associated with OC as well as compare urinary IL-1β levels with clinical parameters.

**Materials and methods**

**Patient cohort**

With prior institutional approval, urine samples were collected from an initial cohort of healthy controls (N=7), women with ovarian benign disorders (N=12), and patients with epithelial OC (N=20) at the H. Lee Moffitt Cancer Center at the University of South Florida (MCC/USF). Table 3.1 shows an age match subset developed from OC patients in the MCC/USF cohort to produce a patient group with an age range (26-92 years and 26-75 age match) more similar to healthy control (37-60 years) and ovarian benign disease (28-81) groups, respectively. The OC category consisted of women diagnosed with OC and primary peritoneal cancer, which is often related to OC. The samples collected from women with ovarian benign disease consisted of broad range of non-malignant gynecologic disorders. The second urine cohort collected with IRB approval at Tampa General Hospital (TGH) consisted of healthy controls (N=3), women with ovarian benign disease (N=11), women with epithelial OC (N=12),
women with other benign gynecologic disease (N=22), and other gynecologic cancers (N=6). Though these cohorts comprise a small pilot study (Table 3.1), they are representative of a typical clinical practice with regards to histology subtypes.

Where possible, H & E sections from paraffin blocks were reviewed to confirm the histologic diagnosis according to FIGO scores. Anonymized information regarding patient age, body mass index (BMI), and tumor type were also obtained as per availability.

**Sample Preparation**

Urine samples were collected from patients, anonymized to protect patient identity, and released from the tissue banks for this research project. All samples were kept in ice following collection. MCC/USF urine samples were treated with a standard protease inhibitor cocktail (80ug/mL 4-(2 aminoethyl)-benzene sulfonyl fluoride, 200ug/mL EDTA, 0.2ug/mL leupeptin, 0.2ug/mL pepstatin, Sigma Scientific, St. Louis, MI). All samples were centrifuged at 3000 × g. Urinary supernates and serum samples were then aliquoted and stored at -20°C.

**Enzyme-linked Immunosorbent assay**

To measure IL-1β levels in patients’ urine and serum, samples were assayed using the quantitative sandwich enzyme-linked immunosorbent assay (ELISA; R&D Systems, Inc., Minneapolis, MN) according to the manufacturer’s instructions. Fluorescence was read on an ELx800 Absorbance Microplate Reader (Biotek, Winooski, Vermont) using Gen5 Data Analysis Software (Biotek, Winooski, Vermont). Resultant values were derived from a standard curve and expressed as the mean IL-1β concentration of triplicate samples ± standard error.
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**Statistical analysis**

Samples for IL-1β ELISA were run in triplicate and the data subjected to descriptive, one way ANOVA, parametric, Kruskal-Wallis, Mann-Whitney U, Spearman correlation, and Wilcoxin W analyses.

**Results**

**Urinary levels of IL-1β decrease with increasing patient age.**

Levels of urinary IL-1β were compared in 93 patients of the entire cohort and 87 patients of the age matched subset. Average urinary IL-1β levels were compared to patient age and as divided by decade grouping. The highest levels of urinary IL-1β were found in the 20-29 and 30-39 age groups in both the entire cohort (Figure 3.1A) and the age matched subset (Figure 3.1B), respectively, suggesting a relationship between increasing age and urinary IL-1β levels.

Levels of serum IL-1β were compared in 19 patients of the entire cohort. In agreement with comparisons between urinary IL-1β levels with age, serum IL-1β levels showed a similar pattern with the highest average serum IL-1β level in the 20-29 age group (Figure 3.1C). This further confirms a possible relationship between age and IL-1β levels. Despite the tendency for increased urinary IL-1β levels in the 20-29 and 30-39 age groups, no statistical differences were found.

**Urinary IL-1β levels are elevated in patients with ovarian cancer.**

The levels of urinary IL-1β were generally negligible (average 0.36 pg/ml) in healthy control samples of the entire cohort and the age match subset, respectively (Figure 3.2A & 3.2B). However, urinary levels from women with benign ovarian disease were increased approximately four-fold compared to healthy controls (average benign 1.26 pg/ml) (Figure 3.2A & 3.2B).
Figure 3.1. Urinary and serum levels of IL-1β are elevated in 20-29 and 30-39 years age groups.
Figure 3.1 Continued. Urinary and serum levels of IL-1β are elevated in 20-29 and 30-39 years age groups. ELISA was utilized to measure IL-1β (mean pg/ml) in the urine and serum of study samples. (A) Urinary levels in all samples (N=92). (B) Urinary levels in age match subset (N=86). (C) Serum levels in available samples. Samples were examined in triplicate and the data expressed as mean.
Figure 3.2. Urinary IL-1β levels are elevated in ovarian cancer patients.
Figure 3.2 Continued. Urinary IL-1β levels are elevated in ovarian cancer patients.
Figure 3.2. Urinary IL-1β levels are elevated in ovarian cancer patients. Urinary samples were analyzed in triplicate by ELISA and data expressed as mean ± standard error in pg/ml. (A) Entire cohort of healthy controls, benign ovarian disorders, ovarian cancer patients, other gynecological benign disorders, and other gynecological cancers; (B) age match subset of healthy controls, benign ovarian disorders, ovarian cancer patients, other gynecological benign disorders, and other gynecological cancers. (C) Non-inflammatory benign disorders and inflammatory benign disorders in all ovarian benign and other gynecological benign disorders. (D) Average age and average urinary IL-1β per diagnosis of healthy (normal) (N=10), benign ovarian disorder (N=25), and ovarian cancer (N=27). Data expressed as mean ± standard deviation of age (in years).

Likewise, urinary levels of IL-1β associated from OC patients exhibited the highest average levels of 1.57 pg/ml IL-1β in the entire cohort (Figure 3.2A) and 1.88 pg/ml IL-1β in the age matched subset (Figure 3.2B), respectively. Levels of urinary IL-1β in other gynecological benign disorders (average 1.56 pg/ml) and other gynecological cancers (average 1.36 pg/ml) were increased in comparison to healthy controls (p ≤ 0.005 and 0.05), respectively, but were decreased in comparison to urinary levels of IL-1β from OC patients (Figure 3.2A & 3.2B).

Average levels of urinary IL-1β were compared in all benign disorder patient samples, including both ovarian benign and other gynecological benign disorder patient samples. The average urinary IL-1β levels were increased in inflammatory benign disorders (1.88 pg/ml) when compared to non-inflammatory benign disorders (0.82 pg/ml) (p = 0.001) (Figure 3.2C). Levels of corresponding urine and serum IL-1β were available from 19 patients of the entire cohort. Paired serum IL-1β showed a similar pattern of increased average levels in the benign ovarian and OC group, respectively, with the highest average levels in the OC group (Figure 3.3). Since there was a significant correlation between serum and urinary IL-1β levels in ovarian benign disorders (r = 0.949, N = 4, p = 0.051) and in OCs (r = 0.724, N = 11, p = 0.012), and there was a very limited number of available serum samples, all remaining clinical comparisons were completed in urine samples only.
When urinary IL-1β levels were analyzed in the age matched cohort with respect to diagnosis and age, the average age in the healthy controls, benign ovarian disorders, and OC patients was 49.3, 52.2, and 57.7 years, respectively (Figure 3.2D). The average urinary levels of IL-1β in these healthy controls, benign ovarian disorders, and OC patients was 0.36 pg/ml, 1.30 pg/ml, and 1.88 pg/ml, respectively (Figure 3.2D) supporting a trend for increased urinary IL-1β levels with disease progression.

Figure 3.3. Urinary and serum IL-1β levels are elevated in ovarian cancer patients. Urinary and serum samples were analyzed in triplicate by ELISA and data expressed as mean ± standard error in pg/ml. Serum and urinary IL-1β in healthy controls, benign ovarian disorders, and OC patients.
Urinary IL-1β levels are increased in patients with a family history of cancer.

Using the age matched sample subset, urinary IL-1β levels were analyzed with respect to a family history of cancer though the BRCA1 status of these patients could not be confirmed. Average urinary IL-1β levels in a healthy control patient with no family history of cancer was higher (0.93 pg/ml) compared to healthy control patients with a family history of cancer (0.27 pg/ml) (Figure 3.4A). In a patient with benign ovarian disease and no family history of cancer, urinary IL-1β levels were 0.33 pg/ml; while average levels were 0.96 pg/ml in benign ovarian patients with a family history of cancer (Figure 3.4A).

Though I could not identify the lack of family history of cancer in most of the OC patients, in those OC patients with confirmed family history of cancer average urinary IL-1β levels were 6.33 pg/ml (Figure 3.4A), while the average urinary IL-1β levels of the remaining OC patients was 1.16 pg/ml. However, when sample 3119-13 that is about 20-fold higher than the other similar samples was excluded from the analyses, the average urinary IL-1β levels decrease to 1.01 pg/ml (Figure 3.4A) and was lower than the remaining OC patients.

Thirteen patient samples of the age matched subset were further narrowed to 8 patients with a first degree family history of ovarian and/or breast cancer. Average levels of urinary IL-1β were higher in patients with a family history and benign ovarian disorder (0.99 pg/ml) or OC (6.33 pg/ml & 1.01 pg/ml) compared to patients with no family history of cancer and diagnosed as being healthy (0.93 pg/ml), having benign ovarian disorders (0.33 pg/ml), or having OC (1.16 pg/ml) (Figure 3.4B).

Urinary IL-1β levels correlate with body mass index (BMI).

Levels of urinary IL-1β were measured in 23 patient samples of the age matched subset and compared with patient BMI.
Figure 3.4. Urinary IL-1β levels correlate with familial history of cancer. Urinary IL-1β was analyzed in triplicate by ELISA and data expressed as mean ± standard error in pg/ml in age match subset. (A) Patients with family history of any cancer. (B) Patients with family history of ovarian and/or breast cancer in first degree family members only.
The classifications of BMI as underweight, normal, overweight, and obese were according to World Health Organization standards (Table 3.2). Patients classified with a normal BMI had the lowest average urinary IL-1β levels of 0.92 pg/ml (Figure 3.5A) while patients classified as overweight had average urinary IL-1β levels of 1.72 pg/ml or about double that found in normal BMI patients (Figure 3.5A). Patients classified as obese had the highest average urinary IL-1β levels of 5.26 pg/ml; however, when samples 3119-2 and 3119-13 were excluding since IL-1β levels were at least 15-fold higher than similar samples, urinary IL-1β levels averaged 1.33pg/ml, still greater than patients with normal BMI (Figure 3.5A). While a single patient classified as underweight had a urinary IL-1β level of 2.20 pg/ml (Figure 3.5A), overall urinary IL-1β levels appeared to increase with increasing BMI.

Further analyses of urinary IL-1β levels with BMI and clinical diagnosis revealed that healthy control patients had an average normal BMI of 22.12 and the lowest average urinary IL-1β level of 0.67 pg/ml (Figure 3.5B). Patients with benign ovarian disease fell within the obese BMI classification (31.94)) and demonstrated a 4-fold higher average urinary IL-1β level of 2.66 pg/ml (Figure 3.5B) in comparison to the control group. OC patients had an average BMI of 29.13 classifying them as overweight and 4.7-fold higher average urinary IL-1β level of 3.15 pg/ml (Figure 3.5B) in comparison to the control group.

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<td>International Classification of BMI</td>
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Figure 3.5. Urinary IL-1β levels are elevated in patients with overweight and obese BMI classification. (A) Urinary IL-1β levels in patient samples in relationship to BMI: Underweight (N=1), Normal (N=11), Overweight (N=2), Obese (N=9). Urinary IL-1β was analyzed in triplicate by ELISA and data expressed as mean ± standard error in pg/ml in age match subset. (B) Average urinary IL-1β levels and average BMI per diagnosis category: Normal (N=2), Ovarian Benign (N=9), Ovarian Cancer (N=11). Data expressed as mean ± standard error of BMI and mean urinary IL-1β in age match subset.
Discussion

While inflammation is an essential biological process for normal development and tissue homeostasis, it is also involved in a number of pathologic conditions including tissue injury, chronic inflammation, immunological diseases, and cancer [10]. Epidemiological studies have shown a link between chronic inflammation and risk for cancer as evidenced by prolonged infection with *Helicobacter pylori* and gastric cancer, inflammatory bowel disease and colon cancer, and prostatitis and prostate cancer [11]. In the ovary, chronic inflammation resulting from repeated ovulatory wounding and repair promotes oxidative stress which enhances DNA replication errors and ultimately, oncogenesis [12]. Inflammation is regulated by several factors that can either promote or inhibit inflammation and since epithelial ovarian cancers (EOCs) are highly inflammatory, this pilot study evaluated urinary levels of the proinflammatory mediator, IL-1β against clinical parameters in order to gain a better understanding of this disease. Data from combined cohorts indicated elevated urinary IL-1β among OC patients compared to healthy controls or women with benign gynecologic disease. Further, this study was able to evaluate four clinical parameters in relation to urinary IL-1β levels: (1) patient age, (2) diagnosis, (3) family history of cancer, and (4) BMI.

When I considered patient age at the time of sample collection, the highest levels of urinary IL-1β levels were found in the 20-29 and 30-39 years age groups followed by declining IL-1β levels as age increased. In keeping with our findings and according to the American Society for Reproductive Medicine (ASRM), a woman’s potential reproductive capacity begins to gradually decline at approximately 32 years of age and more rapidly decreases after 37 years of age [13]. IL-1β has been suggested to play a role in female reproduction; specifically in ovulation and oocyte maturation, and inflammatory-linked mechanisms, such as production and
activation of proteolytic enzymes, prostaglandin production, nitric oxide production, cellular metabolism, and steroidogenesis [14, 15]. Therefore, it seems likely that after 40 years of age, there would be a decrease in urinary IL-1β levels as oocyte maturation and ovulation decrease in preparation for menopause. In contrast, however, Vural, et al. found higher plasma levels of IL-1β in postmenopausal (≥48.6 years) women than in premenopausal (30.5 ± 2.5 years) women with levels of IL-1β decreasing below the premenopausal levels only after hormone replacement therapy [16]. Consequently, though the information was not readily available, perhaps the sharp decrease in urinary IL-1β levels seen in this study in women > 60 years may be due, in part, to the usage of hormone replacement therapy.

Urinary IL-1β levels alone had limited success in differentiating disease status. Urinary IL-1β levels were higher in benign and cancer samples when compared to healthy controls, respectively. IL-1β is present in the serum and ascites of OC patients [9] and has been shown to be involved with cancer tumorigenesis, angiogenesis, and metastasis [17]. The inability of urinary IL-1β to differentiate between benign and malignancy may be confounded by the inflammatory nature of so many benign and cancer conditions. That is, many benign ovarian conditions develop in an inflammatory microenvironment. For instance, endometrioma, a form of endometriosis in the ovary, is a highly inflammatory condition [18] which would expectedly result in high levels of urinary IL-1β [19]. However, proinflammatory markers including serum C-reactive protein (CRP), IL-6, and IL-8 have all been used in clinical studies to differentiate between normal, benign tumor, and OC [20-22]. Immunohistochemical analysis showed differential expression of IL-18 and its receptors in benign ovarian tumors, borderline ovarian tumors, and ovarian carcinomas [23]. Therefore, developing and employing a panel of
inflammatory mediators, including urinary IL-1β, may eventually benefit differential diagnostic and prognostic outcomes of OC.

Patient samples with confirmed family history of cancer were limited in this small pilot study. Family history of cancer tended to relate to elevated urinary IL-1β in patients with ovarian benign disease and a first degree family history of ovarian and/or breast cancer. This supports the recent dualistic model that EOCs arise as either two types: Type 1 and Type 2 [24]. Type 1 tumors may arise in a step-wise progression from a benign precursor lesion such as, the highly inflammatory condition, endometriosis. Further, the prior tissue culture data using OSE cells with endogenous or exogenous BRCA1 185delAG revealed that this germline mutation increased expression and secretion of IL-1β, thereby potentially promoting a chronic inflammatory microenvironment (chapter 2). However, it is important to remember that not all individuals at risk for OC develop the disease so that secondary events, perhaps beyond a FH, may be necessary to promote disease. In keeping, I found minimal urinary IL-1β in healthy controls with a family history of any cancer which would most closely resemble the control cell model of FH IOSE 118 + PCDNA3.1 (chapter 3).

Obesity as a risk factor of OC is controversial. Recently, a meta-analysis of 47 epidemiological studies found increased OC risk with high BMI [25]. The Ovarian Cancer Association Consortium investigated 15 case-control studies and found overweight and obese women were associated with increased risk of OC [26]. The National Institutes of Health also found that BMI was significantly associated with increased risk in women who never used hormone therapy [27]. Canchola, et al. found a positive association between OC risk and adult weight gain, waist circumference, and waist-to-hip ratio but no association to overall obesity as
classified by BMI [28]. In agreement, high waist-to-hip ratio was also associated with increased risk of OC but no association with BMI was found [29].

Contradictory, some studies have found no relationship between obesity and risk of OC. Schouten, et al. reported no association overall between BMI and risk of OC; however, they did report a positive association among premenopausal women, but not in postmenopausal women [30]. More recent prospective studies reported no significant relationship between BMI and OC risk, irrespective of menopausal status [31, 32]. There was also no evidence for risk of OC and weight or weight gain among BRCA1 or BRCA2 mutation carriers [33]. Interestingly, Engleland et al. found the risk of OC was not associated with adult BMI, but suggested a possible increased risk in women who were obese in young adulthood [34].

In this study, one of the most apparent clinical features related to elevated urinary IL-1β was BMI. I found increased urinary IL-1β levels associated with higher BMI. Patients classified as overweight and obese were also likely to be diagnosed with OC and ovarian benign disorders, respectively, while concomitantly demonstrating the highest average urinary IL-1β levels. In contrast, healthy controls with normal BMI exhibited the lowest average urinary IL-1β. Among the data was a single patient case classified as underweight, but with elevated urinary IL-1β levels and a diagnosis of OC. It is tempting to speculate that this individual may have had advanced OC at the time of sample collection where anorexia, nausea, and weight loss due to the increased glucose, lipid, and protein requirements of the tumor [35] could manifest as low BMI compounded with elevated urinary IL-1β as a result of advanced disease.

The obesity and elevated IL-1β levels found in OC patients may contribute to OC mortality. Studies have shown no association between pre-diagnosis (average 5 - 10 years prior to OC diagnosis) BMI and OC mortality [36, 37], but have also suggested obesity is associated
with poor outcome [38]. These contradictory findings may suggest that obesity itself is not the factor leading to increased OC mortality, but perhaps obesity acts as a comorbidity factor. For instance, one factor that may contribute to poor outcome is the difficulty of proper chemotherapy dosages for obese patients. A study of dosing practices of clinicians found a significant proportion of advanced OC patients were overweight or obese, as seen in the current study, and under-dosing of chemotherapy in obese populations was common even though this did not affect overall survival [39]. The variability in dosing when prescribing chemotherapy is largely due to concern for potential over-dosing and chemotherapy associated toxicities [40]. However, one may speculate dosing variability due to patient obesity may add to a patient’s poor outcome since the tumor is not getting the proper chemotherapy dose. High mobility group A2 (HMGA2) is a protein that can regulate transcription by altering chromatin architecture and facilitate the assembly of multiprotein complexes of transcriptional factors [41] and is found expressed in serous OC tumors and not in normal ovarian epithelial cells [42]. OC tumors with high expression of HMGA2 and high BMI negatively affected overall survival; and high BMI negatively influenced the response to chemotherapy treatment [43].

Furthermore, obesity may increase tumor aggressiveness. Increased metabolic activity and glucose concentrations are associated with highly aggressive OC cell lines [44]. The changes in metabolism and increase in glucose concentrations may occur as a result of the Warburg effect. In cancer, the Warburg effect is regarded as a characteristic metabolic process that may contribute to cell survival in a stressful environment, such as the stress of chronic inflammation [45]. The Warburg effect suggests that cancer cells produce energy predominately by glycolysis and lactic acid production over oxidative phosphorylation [46]. An in vivo mouse model demonstrated increased tumor size with obesity and the tumors in the obese mice had a
unique molecular makeup with upregulated inflammation genes [47]. Obesity in OC patients may be further exacerbating the disease by contributing to an inflammatory environment. Obesity-related type 2 diabetes is associated with chronic inflammation [48-50] and IL-1β levels have been shown to be correlated with obesity and obesity related disorders. Individuals with combined elevated plasma levels of IL-1β and IL-6 were at an increased risk for developing type 2 diabetes [51]. Obese patients who underwent even mild weight loss had a 45% decrease in serum IL-1β levels over a three-year study period [52]. Leptin, an adipocytokine involved in the pathogenesis of insulin resistance necessary for developing type 2 diabetes, induces β-cell apoptosis and impaired β-cell function by promoting IL-1β production in human pancreatic islets [53]. Expression of leptin is also positively correlated with BMI [54]. In this study, obese patients may exhibit the highest urinary IL-1β levels as a result of obesity and the increased urinary IL-1β may be indicative of advanced disease.

The greatest limitation of urinary IL-1β as a biomarker for OC is kidney function. Inflammatory mediators, including IL-1β, are typically found elevated in the urine and serum of patients with impaired kidney function [55-57]. Furthermore, elevated levels of IL-1β have been reported in vaginal secretions associated with gynecologic infections; however, Basso et al were unable to detect IL-1β in patient urine or serum [58]. Interestingly, two samples in our study, 3119-2 and 3119-13, displayed unusually elevated urinary IL-1β in comparison to their counterparts. Such marked urinary IL-1β levels may be attributed to possible kidney injury or gynecological infection.

The data in this chapter has been derived from a small pilot study. However, the sample size of this study is in keeping with other published pilot studies examining IL-1β in urine, serum, and plasma [16, 55, 57-60]. My study had limited statistical significance which may be
associated to the small sample size. Furthermore, a normal baseline value for urinary IL-1β in women has not yet been established in the literature [61]. Therefore, future study and increased sample size may assist in the development of baseline and threshold values that could be used to differentiate between healthy, benign disorders, OC, as well as other clinical parameters such as metabolic disruption. Lastly, this study also supports the theory of elevated urinary IL-1β being associated with cancer progression such that the identification of an inflammatory profile specific to EOC may benefit non-invasive diagnostic and prognostic applications as well as lead to the development of adjuvant therapies utilizing target-specific anti-inflammatory treatments to reduce the mortality associated with this disease.

References


Chapter 4:

Molecular Characterization and Comparison of wtBRCA1 and Founder Mutations, 

BRCA1 185delAG and BRCA1 5382insC

Introduction

The breast cancer susceptibility gene 1 (BRCA1) is a tumor suppressor that when mutated is associated with increased breast and ovarian cancer (OC) risk [1, 2]. The best characterized BRCA1 germline mutations are the founder mutations, BRCA1 185delAG and 5382insC [3, 4]; and these mutations are associated with the majority of OC cases in the Ashkenazi Jewish community.

While these mutations are not susceptible to nonsense-mediated messenger RNA (mRNA) decay suggesting that viable mutant proteins could be produced [5, 6], these protein products have not been isolated in vivo or in vitro. The difficulty of locating these mutant protein products by antibody is made especially difficult due to their homology with wild-type BRCA1 (wtBRCA1). Therefore, to date, all studies of the BRCA1 185delAG employed PCR confirmation of the mutation and all functional changes seen in mutation carrying cell lines are believed to be the result of a novel truncated protein (yet to be visualized by protein study). Even so, my lab has described several functions of the BRCA1 185delAG mutation, including enhanced apoptosis in response to chemotherapy treatment [7, 8], increased expression of adhesion and metastasis proteins (Linger in process), and increased expression of
proinflammatory mediators (chapter 2). In order to better understand the function of the predicted BRCA1 185delAG truncated protein, BRAT, I produced the BRCA1 185delAG mutated protein that can be utilized in future functional studies. I also describe potential characteristics of the putative BRCA1 185delAG and 5382insC mutation proteins based on amino acid sequence characteristics.

Materials and Methods

Plasmid Construction and Transfection

cDNA encoding for BRCA1 185delAG with an N-terminal His-tag and C-terminal S-protein motif was generated by Midland Certified Reagent Company (Midland, TX) using the published genbank BRCA1 sequence U14680. S-His-BRAT cDNA was then ligated using T4 DNA ligase (New England Biolabs) in 1X T4 ligase buffer (50mM Tris-HCl (pH7.5), 10 MgCl₂, 10mM dithiothreitol, 2mM ATP, 25ug/ml BSA) into the pTriEx-4 plasmid (Novagen), transformed into DH5α competent cells and isolated under standard conditions (Invitrogen). The S-His-BRAT plasmid sequence was confirmed.

Protein Expression and Immunoprecipitation

DH5α competent cells transformed with S-His-BRAT cDNA were grown in 3 ml LB broth with ampicillin (100mg/L) overnight at 37°C, shaking at 250 rpm. The next day, the microbial culture was transferred into 1L of LB broth with ampicillin (100mg/ml) shaking at 250 rpm, at 37°C. When A600 reached 0.7-1, 0.2 g/ml stock IPTG was added at a concentration of 0.2g/L to induce protein expression. The culture was grown overnight at 37°C shaking at 250 rpm. Cells were harvested and washed in PBS by centrifugation at 4°C for 15 minutes at 2500×g. Cell pellet was incubated with 3 ml ice cold RIPA buffer at 4°C for 10 minutes and
cells were disrupted by repeated aspiration through a 21 gauge needle. Cell lysate was incubated with 2 ug of primary antibody for 1 hour at 4°C. Protein A/G agarose suspension was added to lysate and antibody mixture followed by incubation at 4°C on a rocker overnight. The suspension was collected by centrifugation at 500 x g for 2 minutes at 4°C. The cell pellet was washed with RIPA buffer containing protease inhibitors followed by analysis for IP confirmation of 185delAG production and isolation by western blot as described in chapter 2.

Simultaneous SDS-PAGE analysis was conducted. The SDS-PAGE was stained with coomassie blue and the visualized 185delAG protein band was excised and amino acid sequenced at the H. Lee Moffitt Cancer Center and Research Institute. Antibodies: His tag (27E8) (1:1000) Cat. #2366S Cell Signaling Technology (Beverly, MA) and BRCA1 (Ab-1) (1:1000) Cat. # OP92 Calbiochem (Billerica, MA).

**Peptide Mapping**

Following in-gel tryptic digestion, peptides were extracted and concentrated under vacuum centrifugation. A nanoflow liquid chromatograph (Easy-nLC, Proxeon, Odense, Denmark) coupled to an electrospray ion trap mass spectrometer (LTQ, Thermo, San Jose, CA) was used for tandem mass spectrometry peptide sequencing experiments. The sample was first loaded onto a trap column (BioSphere C18 reversed-phase resin, 5µm, 120Å, 100 µm ID, NanoSeparations, Nieuwkoop, Netherlands) and washed for 3 minutes at 8 µL / minute. The trapped peptides were eluted onto the analytical column, (BioSphere C18 reversed-phase resin, 150mm, 5µm, 120Å, 100 µm ID, NanoSeparations, Nieuwkoop, Netherlands). Peptides were eluted in a 60 minute gradient from 5% B to 45% B (solvent A: 2% acetonitrile + 0.1% formic acid; solvent B: 90% acetonitrile + 0.1% formic acid) with a flow rate of 300 nl/min. Five tandem mass spectra were collected in a data-dependent manner following each survey scan.
Sequences were assigned using Mascot (www.matrixscience.com) searches against human International Protein Index entries. Carbamidomethylation of cysteine, methionine oxidation, and deamidation of asparagine and glutamine were selected as variable modifications, and as many as 2 missed tryptic cleavages were allowed. Precursor mass tolerance is set to 2.5 and fragment ion tolerance to 0.8. Results from Mascot were compiled in Scaffold, which was used for manual inspection of peptide assignments and protein identifications.

**Computational Characterization of Disorder**

The distribution of intrinsic disorder propensity in BRCA1, 185delAG, and 5382inC was analyzed using three algorithms, PONDR® VLXT [9], PONDR® VSL2 [10], and PONDR-FIT [11]. PONDR® VLXT is capable of identifying potential molecular interactions motifs in disordered proteins and disordered protein regions [12, 13]. PONDR® VSL2 more accurately predicts general protein disorder [10], whereas PONDR-FIT is a meta-predictor that combines six individual predictors [11], such as PONDR® VLXT [9], PONDR® VSL2 [10], PONDR® VL3 [14], FoldIndex [15], IUPred [16], and TopIDP [17]. This meta-predictor is moderately more accurate than each of the component predictors and provides accurate disorder predictions at the residue level, which allows evaluation of the number and size of the predicted disordered protein segments.

**Evaluation of the Aggregation Propensity**

Prediction of aggregation-prone regions within the protein of interest was achieved utilizing the consensus web tool, AmylPred2 (http://aias.biol.uoa.gr/AMYLPRED2/) [18]; which includes the following methods: Aggrescan [19], AmyloidMutants [20], Amyloidogenic Pattern [21], Average Packing Density [22], Beta-strand contiguity [23], Hexapeptide Conformational Energy [24], NetCSSP [25], Pafig [26], SecStr (Possible Conformational
Switches) [27], Tango [28], and Waltz [29]. AmylPred2 recognizes amyloid structures; a specific subset of insoluble fibrous protein aggregates [18]. Web tool PASTA2.0 (http://protein.bio.unipd.it/pasta2/) was also utilized to predict aggregation-prone regions; as well as, intrinsic disorder and secondary structure [30].

**Prediction of Post-Translational Modifications**

Ubiquitination sites were predicted using the UbPred predictor [31]. UbPred is a random forest predictor with an accuracy of 72%. Sumoylation sites were predicted using SUMOplot™ Analysis Program (Abgent Tools) (http://http://www.abgent.com/sumoplot). Phosphorylation and glycosylation sites were predicted using HMMpTM (http://http://aias.biol.uoa.gr/HMMpTM/index.php) [32].

**ANCHOR Analysis**

Potential protein binding sites were identified by the ANCHOR algorithm [33, 34]. This algorithm is based on the hypothesis that long regions of disorder contain localized potential binding sites that cannot form enough favorable intra-chain interactions to fold on their own, but are likely to gain stabilizing energy by interacting with a globular protein partner. The PredZinc version 1.4 server was utilized to predict possible zinc binding domains in the amino acid sequence [35].

**Signal Peptide Analysis**

The SignalP 4.1 server was utilized to predict the presence and location of secretory pathway signal peptide cleavage sites in the amino acid sequence of the target protein [36]. This method incorporates a prediction of cleavage sites and a signal peptide/non-signal peptide prediction based on a combination of several artificial neural networks.
Secondary Structure

Prediction of the secondary structure for the target protein was determined by GOR IV (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html) [37] and PORTER (http://distill.ucd.ie/porter/) [38]. These programs analyze multiple sequence alignments and the highest probability of each secondary structure at each amino acid position. GOR IV produces the highest probability compatible with a predicted helix segment of at least four residues and a predicted extended segment of at least two residues.

Domain Homologue Analysis

SBASE (http://www.icgeb.trieste.it/sbase) is a web tool that encompasses protein domain sequences to facilitate the detection of domain homologues [39]. This tool utilizes sequence alignment to find similar amino acid domains that are of biological significance to the structure and/or function of a target protein. National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) was utilized to align amino acid sequences in order to find sequence similarities (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

BRCA1 185delAG protein produced in competent E. coli cells was isolated by immunoprecipitation.

There was no His-tagged protein in the expected 5kDa range for the 185delAG protein. However, a His-tagged protein in the 10kDa to 20kDa range was produced and isolated from competent E. coli cells by immunoprecipitation as seen by western blot (Figure 4.1A). The resulting protein amino acid sequence from an offsite protein sequencing core at H. Lee Moffitt Cancer Center Proteomics Core confirms BRCA1 185delAG (Figure 4.1B). The expected
protein size of the 38 amino acid BRCA1 185delAG is about 5kDa and was compared to the 208kDa wtBRCA1 and 204kDa BRCA1 5382insC (Table 4.1).

**BRCA1 185delAG is a structurally ordered protein.**

Multiple prediction software programs were utilized to identify molecular characteristics of wild-type BRCA1 (wtBRCA1), BRCA1 185delAG, and BRCA1 5382insC as summarized in Table 4.1. The amino acid sequence of each protein target was utilized to predict the stability of their respective tertiary structure. wtBRCA1 had regions of order and disorder throughout the protein sequence (Figure 4.2A). The PONDR Score was below the standard threshold and confirmed wtBRCA1 as a disordered protein (Figure 4.2B). Protein mean hydropathy, an average of the values that are assigned to amino acids of a target protein representing the hydrophobic or hydrophilic properties of its’ side-chain [40], was also investigated. wtBRCA1 was predicted to have a low mean hydropathy and a low overall protein charge suggesting wtBRCA1 is a hydrophilic protein and was further confirmed to be disordered (Figure 4.2C).

By contrast, BRCA1 185delAG was predicted to be a highly ordered protein with two small regions of disorder in the N and C-terminals (Figure 4.3A & B). The PONDR Score fell above the standard threshold and confirmed 185delAG as an ordered protein (Figure 4.3C). The high mean hydropathy and a low overall net protein charge suggested that it is a hydrophobic protein and further confirmed BRCA1 185delAG as an ordered protein (Figure 4.3D). BRCA1 5382insC had regions of order and disorder as seen in wtBRCA1 (Figure 4.4A). The PONDR Score was below the standard threshold and confirmed 5382insC as a disordered protein (Figure 4.4B).
Figure 4.1. BRCA1 185delAG protein expression and peptide mapping. (A) Western blot visualization of BRCA1 185delAG protein isolated by immunoprecipitation with His tag antibody. (Left) BRCA1 antibody western blot with ladder in lane 1 and immunoprecipitation in lanes 2 and 3. (Right) His tag antibody western blot with ladder in lane 1 and immunoprecipitation in lanes 2 and 3. Black arrow shows band excised for amino acid sequencing. Blue arrow shows expected size of 185delAG ~5kDa. (B) Peptide mapping results from H. Lee Moffitt Cancer Center Proteomics Core.
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<th>wtBRCA1</th>
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<tr>
<td>Predicted size</td>
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<tr>
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<tr>
<td>Ubiquitination Sites</td>
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<td>1</td>
<td>66</td>
</tr>
<tr>
<td>Signal Peptides</td>
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</tr>
<tr>
<td>Amyloid Formation Sites</td>
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<td>12 (31%)</td>
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(\% of amino acid sequence)
Figure 4.2. Predicted intrinsic disorder and hydrophobicity of wtBRCA1. PONDR analysis of wtBRCA1 showed locations of intrinsically ordered and disordered regions in protein sequence (A) and overall protein classification (B & C).
Figure 4.3. Predicted intrinsic disorder and hydrophobicity of BRCA1 185delAG. PONDR analysis of BRCA1 185delAG showed locations of intrinsically ordered and disordered regions in protein sequence (A & B) and overall protein classification (C & D).
Figure 4.4. Predicted intrinsic disorder and hydrophobicity of BRCA1 5382insC. PONDR analysis of 5382insC showed locations of intrinsically ordered and disordered regions in protein sequence (A) and overall protein classification (B & C).
BRCA1 5382insC was predicted to have a low mean hydropathy and a low overall protein charge suggesting BRCA1 5382insC is a hydrophilic protein and was further confirmed to be disordered (Figure 4.4C).

**BRCA1 185delAG has no potential protein or zinc binding sites.**

The potential protein binding sites for wtBRCA1, BRCA1 185delAG, and BRCA1 5382insC were identified by prediction software, ANCHOR. wtBRCA1 had several potential protein binding regions throughout the length of the protein sequence (Figure 4.5A). BRCA1 5382insC also had several potential protein binding regions in a pattern similar to wtBRCA1 (Figure 4.5B). BRCA1 185delAG had no potential protein binding regions and also had no predicted zinc binding domains (Figure 4.5C, D), while wtBRCA1 and 5382insC showed potential zinc binding domains (Figure 4.5E, F).

**BRCA1 185delAG has limited potential post-translational modification sites.**

I analyzed for the presence of potential post-translational modification sites, phosphorylation, glycosylation, ubiquitination, sumoylation, and secretory pathway signal peptides, in each protein target (Table 4.1). All three protein targets contained potential phosphorylation and ubiquitination sites. However, while wtBRCA1 and BRCA1 5382insC had potential glycosylation and sumoylation sites, BRCA1 185delAG had no potential glycosylation or sumoylation sites. Each protein target, wtBRCA1 (Figure 4.6A), BRCA1 5382insC (Figure 4.6B), and BRCA1 185delAG (Figure 4.6C), had no potential signal peptides.

**BRCA1 185delAG is potentially highly self-aggregated.**

Protein self-aggregation software was used to predict the aggregation tendencies of each protein target. Both wtBRCA1 (Figure 4.7A) and BRCA1 5382insC (Figure 4.7B) had a few potential sites of amyloid formation/self-aggregation.
Figure 4.5. Potential protein binding and zinc binding domains.
Figure 4.5. Continued. Potential protein binding and zinc binding domains. ANCHOR analysis showed the locations of potential protein binding domains of wtBRCA1 (A), BRCA1 5382insC (B), and BRCA1 185delAG (C). (D). PredZinc analysis showed the lack potential zinc binding domains in BRCA1 185delAG. (E). PredZinc analysis showed presence of zinc binding domains in wtBRCA1. (F). PredZinc analysis showed presence of potential zinc binding domains in BRCA1 5382insC.
A.

Figure 4.6. Potential signal peptides.

B.
Figure 4.6 Continued. Potential signal peptides. SignalP 4.1 server predicted the presence of signal peptides in wtBRCA1 (A), BRCA1 5382insC (B), and BRCA1 185delAG (C).

A.

<table>
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<th>% disorder</th>
<th>% α-helix</th>
<th>% β-strand</th>
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B.

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C.

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<td>67.89</td>
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<td>42.11</td>
</tr>
</tbody>
</table>

Figure 4.7. Prediction of amyloid formation. PASTA2.0 predicted the number of potential amyloid formation sites in wtBRCA1 (A), BRCA1 5382insC (B), and BRCA1 185delAG (C).
These proteins had only 20 sites of potential amyloid formation, about 1% of the entire amino acid sequence. These sparing potential sites of self-aggregation led to the classification of “No” in Table 4.1 under the “Self-Aggregate” category. BRCA1 185delAG (Figure 4.7C) had 12 potential amyloid formation sites, about 31% of the entire amino acid sequence. Analysis of which 185delAG residues would potentially bind during self-aggregation showed the central, highly ordered region would be the likely location of aggregation, while the more disordered regions in the N and C-terminals showed no potential of aggregation (Figure 4.8A-C).

**BRCA1 185delAG secondary structure is mostly alpha helical.**

The secondary structure composition of each protein target was analyzed. Each target was comprised of alpha helix, extended strands, and random coil. The wtBRCA1 (Figure 4.9A) and BRCA1 5382insC (Figure 4.9B) had similar secondary structure composition. The wtBRCA1 and BRCA1 5382insC both had 32% alpha helix structures; wtBRCA1 had 13% extended strand and BRCA1 5382insC had 12% extended strand; and, wtBRCA1 had 55% random coil and BRCA1 5382insC had 56% random coil. BRCA1 185delAG (Figure 4.9C) was mostly composed of alpha helix (45%), followed by 42% random coil, and 13% extended strand.

In BRCA1 185delAG, the area with the greatest potential for aggregation is also composed of the alpha helix conformation, while the random coil and extended strands are located in areas of disorder with less potential for aggregation (Figure 4.10 A, B, C). The potential protein schematic showed that the BRCA1 185delAG protein is organized with random coil at the N-terminus that leads to an alpha helix structure followed by a second small region of random coil and ending with extended strand and random coil (Figure 4.10D).
Figure 4.8. Prediction of combined amyloid formation and aggregation. (A). PASTA 2.0 predicted self-aggregation at each amino acid residue of 185delAG. (B). Illustrative schematic of 185delAG self-aggregation with the location of amino acids aggregating at blue dots. (C). Graph predicting aggregation in terms of ordered regions and disordered regions of 185delAG.
A.

Sequence length : 1863
GOR4 :
  Alpha helix (Hh) :  592 is 31.78%
  3_10 helix (Gg) :  0 is 0.00%
  Pi helix (Ii) :  0 is 0.00%
  Beta bridge (Bb) :  0 is 0.00%
  Extended strand (Ee) :  241 is 12.94%
  Beta turn (Tt) :  0 is 0.00%
  Bend region (Ss) :  0 is 0.00%
  Random coil (Cc) : 1030 is 55.29%
  Ambiguous states (?) :  0 is 0.00%
  Other states :  0 is 0.00%

B.

Sequence length : 1828
GOR4 :
  Alpha helix (Hh) :  589 is 32.22%
  3_10 helix (Gg) :  0 is 0.00%
  Pi helix (Ii) :  0 is 0.00%
  Beta bridge (Bb) :  0 is 0.00%
  Extended strand (Ee) :  224 is 12.25%
  Beta turn (Tt) :  0 is 0.00%
  Bend region (Ss) :  0 is 0.00%
  Random coil (Cc) : 1015 is 55.53%
  Ambiguous states (?) :  0 is 0.00%
  Other states :  0 is 0.00%

C.

|   |   |   |
|--------------------------------|
| MDLSALRVEEVQNVINAMQKILVSHLSGVDQGTCLHKV |
| ccccccccccccccccccccccccccccccccccccccceeeeee |

Sequence length : 38
GOR4 :
  Alpha helix (Hh) :  17 is 44.74%
  3_10 helix (Gg) :  0 is 0.00%
  Pi helix (Ii) :  0 is 0.00%
  Beta bridge (Bb) :  0 is 0.00%
  Extended strand (Ee) :  5 is 13.16%
  Beta turn (Tt) :  0 is 0.00%
  Bend region (Ss) :  0 is 0.00%
  Random coil (Cc) :  16 is 42.11%
  Ambiguous states (?) :  0 is 0.00%
  Other states :  0 is 0.00%

**Figure 4.9. Secondary structure composition.** GOR4 server predicts the secondary structure composition of wtBRCA1 (A), BRCA1 5382insC (B), and BRCA1 185delAG (C).
Figure 4.10. Distribution of secondary structure throughout BRCA1 185delAG protein. PASTA2.0 analysis of BRCA1 185delAG graphs the location of alpha helix (A), extended strand (B), and random coil (C) along with predicted location of aggregation. (C). Illustrative diagram of secondary configuration.
**BRCA1 185delAG resembles Peptidase M20.**

In order to understand the function of BRCA1 185delAG in the cell, I analyzed the amino acid sequence using SBASE prediction software to identify other similar proteins. Analysis of domain similarities in the BRCA1 185delAG and other proteins showed a predicted Peptidase M20-like domain (Figure 4.11). Alignment of the BRCA1 185delAG amino acid sequence to the Peptidase M20 amino acid sequence showed the N and C-terminals of BRCA1 185delAG were the domains of similarity (Figure 4.12).

**Discussion**

While founder mutations, BRCA1 185delAG and BRCA1 5382insC, are associated with increased risk of OC, the molecular characterization of their potential protein products has not been reported. Therefore, I sought to begin early characterization of these proteins to better understand their role in OC oncogenesis. Our previous studies have focused on the BRCA1 185delAG mutation; however, I wanted to compare this mutation to the BRCA1 5382insC mutation to investigate what characteristics may be shared by founder mutations and which are distinct to the BRCA1 185delAG. Overall, the study results showed the BRCA1 185delAG protein product is structurally and molecularly distinct from the wtBRCA1 while BRCA1 5382insC more closely resembles wtBRCA1.

Predictably, the BRCA1 185delAG mutation lost many of the post-translational modification sites since it has lost the majority of the wtBRCA1 protein. In contrast, BRCA1 5382insC has retained many of the potential post-translational modification sites since it has retained much of the wtBRCA1 protein. Furthermore, both BRCA1 185delAG and BRCA1 5382insC had no potential secretory signal peptides similar to wtBRCA1.
**Figure 4.11. BRCA1 185delAG contains a Peptidase M20-like domain.** SBASE server analysis of BRCA1 185delAG.

**Figure 4.12. Peptidase M20 and BRCA1 185delAG sequence alignment.** BLAST shows regions and corresponding amino acid sequence of BRCA1 185delAG with commonality to Peptidase M20.
Since neither mutation gained the attributes needed to independently enter the secretory pathway, it is likely that both mediate oncogenic changes within the cell and not by extracellular receptor binding. wtBRCA1 is located in and functions mainly in the nucleus, so one might speculate that BRCA1 185delAG and BRCA1 5382insC also function within the nucleus. The presence of BRCA1 185delAG and BRCA1 5382insC in the nucleus may allow them to independently influence nuclear processes such as, DNA replication, cell cycle signaling, and transcription factor recruitment as seen in wtBRCA1 [2]. wtBRCA1 has two nuclear localization signals (NLSs) [41, 42] and a nuclear export signal (NES) [43] that allows it to shuttle between nuclear and cytoplasmic compartments [44, 45]. Potentially, the BRCA1 5382insC may also shuttle in and out of the nucleus since the NLS and NES domains are still intact. However, the NLS and NES domains are lost in the BRCA1 185delAG, so it is unlikely that this mutation can independently transport out of the nucleus and is in keeping with our earlier report that BRCA1 185delAG is located within the nucleus [7].

Ordered proteins adopt energetically stable three-dimensional conformations with minimum free energy based upon the sequence of the amino acids in their polypeptide chain. More recently, a new category of proteins has been discovered known as intrinsically disordered proteins (IDPs) which cannot adopt an energetically favorable three-dimensional conformation, due to their unique amino acid sequence arrangement; and, therefore, lack a stable tertiary structure in vitro [46]. Their dynamic tertiary structure allows IDPs to be involved in multiple functional pathways, conferring multiple regulatory functions [47-49]. Mutations in and dysregulation of IDPs has been associated with many diseases, including cancer [49-51]. The tumor suppressor p53 is an IDP and functions via its C-terminal intrinsically disordered region (IDR) that simultaneously exists in differing three-dimensional conformations that each have
unique functions [50]. Phosphatase and tensin homologue (PTEN) is a frequently mutated tumor suppressor that governs many cellular processes including survival, proliferation, energy metabolism, and cellular architecture [52]. PTEN deficiency or dysregulation drives endometrial, prostate, brain and lung cancers and causes neurological defects [53, 54]. PTEN has been identified as an IDP with an IDR in the C-tail which is known to regulate the majority of PTEN functions and protein interactions [55].

BRCA1 has been shown to be an IDP with the majority of the central region as its IDR, which is able to mediate interactions with DNA and p53 [56]. BRCA1 is also a hydrophilic protein which would allow for enhanced interactions with other proteins as evident by the many potential protein binding sites seen in this study [57-63]. Conversely, the BRCA1 185delAG mutation is a very ordered, hydrophobic protein that showed no potential protein binding sites. BRCA1 185delAG is as an example of an IDP (BRCA1) mutated to an ordered protein (185delAG) and subsequently being associated with increased cancer risk. Further, study is clearly warranted to determine if transition of BRCA1 via mutation from disorder to order could be utilized as predictor of the likelihood of oncogenic transformation in specific mutations.

Interestingly, the highly hydrophobic BRCA1 185delAG with no potential protein binding partners seems to have a high potential to self-aggregate. This may explain the previous difficulty our lab has encountered in attempting to isolate this protein in vitro from ovarian surface epithelial cells. Also, the isolated His tagged protein in this study was larger than the expected size of the protein and self-aggregation would explain this phenomenon. Perhaps self-aggregation is necessary for its’ deleterious properties as seen in other diseases. Protein aggregation can result in a variety of neurodegenerative and non-neuropathic disorders such as Huntington’s disease, Parkinson’s disease, thrombosis and type 2 diabetes [64]. Aggregation of
gain of function mutant p53 has also been shown to contribute to the development of cancer [65].

α1-Antitrypsin deficiency (AATD) is a genetic disorder that can lead to chronic obstructive pulmonary disease (COPD) in adults and/or liver disease in infants, children, and adults [66]. It is characterized by the pathogenic polymerization of α1-antitrypsin protein that induces cellular damage by large aggregates [66]. The potential self-aggregation of BRCA1 185delAG may induce cellular damage resulting in the development of chronic inflammation as seen in chapter 2 by the increased endogenous production of IL-1β and the possibility of oncogenic transformation.

Unanticipated, I found that BRCA1 185delAG contains an alpha helical secondary structure which is an uncommon trait of self-aggregating proteins. Self-aggregating proteins are mainly composed of beta sheets [67]; although, some studies have suggested an intermediate step in amyloid formation that consists of an alpha helix to beta sheet folding transition [68, 69]. BRCA1 185delAG is a very small protein of only 38 amino acids making it unlikely to fold into a β-sheet structure during aggregation.

In an attempt to begin to understand the functions of BRCA1 185delAG in the cell, I looked to proteins with similar amino acid sequences and found a resemblance to the Peptidase M20 family. This is a group of proteins that contains metallopeptidases and non-peptidase homologues or amidohydrolases that belong to the MEROPS peptidase family M20 [70]. These peptidases have two catalytic zinc ions at the active site. While wtBRCA1 has a RING finger domain that utilizes the binding of two zinc ions [2], the BRCA1 185delAG mutation has lost this domain [71] and zinc binding analysis of BRCA1 185delAG showed that no potential zinc binding properties were retained. Consequently, it is unlikely that BRCA1 185delAG functions in a metallopeptidase-like manner. However, a secondary function of the Peptidase M20 family
of proteins is amidohydrolase. The amidohydrolase superfamily is a group of enzymes that undergo catalytic reactions involving amino acids, sugars, nucleic acids, and organophosphate esters [72]. The amidohydrolase active site contains a metal ion including zinc, iron, copper, and calcium [72]. Histone deacetylase (HDAC) is an example of a zinc-dependent amidohydrolase which functions to catalyze the removal of an acetyl group from DNA in the nucleus by cleaving a non-peptide amide bond resulting in a more positively charged DNA molecule which then more tightly coils around histones making DNA less available for transcription [73]. It is tempting to speculate that the BRCA1 185delAG could possibly act in a similar fashion to inhibit transcription of target genes that have tumor suppressor qualities which may be facilitated by the binding of a non-zinc metal ion to the IDRs of the N or C-terminals.

This study is the first examination of the biochemical, molecular and structural composition of the putative BRCA1 185delAG truncation protein. The BRCA1 185delAG protein is structurally and molecularly distinct from wtBRCA1 and fellow founder mutation, BRCA1 5382insC. To date, the protein product of BRCA1 185delAG has not been isolated. I was able to produce, isolate, and confirm the sequence of the protein. Allowing for further protein analysis, such as confirmation of its 3D protein structure, self-aggregation, helical folding and possible protein-DNA interactions leading to a better understanding of its’ oncogeneic functions.

References


Chapter 5:

Concluding Remarks

This study examined the molecular influence of the BRCA1 founder mutation, 185delAG, in normal OSE. I found that the BRCA1 185delAG mutation confers a potentially unique premalignant condition that implicates a role for inflammation in ovarian transformation by altering the expression of the proinflammatory mediator, IL-1β. Enhanced expression of IL-1β could result in a state of chronic inflammation that when coupled with rapid ovulatory-dependent OSE cell division directly or indirectly through downstream targets, IL-6 and IL-8, promotes genetic instability leading to malignant transformation. In agreement, this study showed enhanced levels of urinary and serum IL-1β in OC patients supporting an inflammatory OC environment. In addition to suggesting how the BRCA1 185delAG may increase risk for OC, my data also have implications for OC metastasis, emergence of drug-resistant disease, and unique treatment options, respectively.

Inflammation present in OC may promote metastasis. An in vivo mouse model of spontaneous metastasis demonstrated that tumor cell dissemination from the ovaries correlated temporally with enhanced peritoneal inflammation and depletion of peritoneal macrophages reduced metastasis suggesting a macrophage-mediated mechanism of inflammation [1]. Macrophage-secreted TNF-α, a proinflammatory mediator, is also able to increase tumor cell
invasiveness [2] while adipocyte-secreted proinflammatory cytokines, IL-6 and IL-8, attracted OC cells to the omentum.

Further, OC metastasis is defined by an epithelial-mesenchymal transformation (EMT). EMT is characterized by the disruption of cellular junctions and loss of cellular polarity resulting in morphologic modulation into a fibroblastic phenotype accompanied by increased cell motility and invasion [3]. Embryonically, EMT contributes to gastrulation, and in the ovary to gonadal development [4]. EMT and subsequent mesenchymal-epithelial (MET) transitions associated with alterations of E-cadherin expression [5] are among the most dramatic examples of OSE plasticity in response to its microenvironment and represent critical steps in ovarian tumor progression. Owing to the mesodermal origin of OSE, normal OSE express N-cadherin, but not E-cadherin. However, in contrast to loss of E-cadherin expression with tumor progression in most epithelial cancers, E-cadherin is often re-expressed in OSE lining crypts and inclusion cysts as well as in benign, borderline and primary OCs [5]. Importantly, given that OC metastasizes by shedding into the peritoneal cavity as single cells or cell clusters, transient E-cadherin re-expression maintains OC aggregate formation and survival in the peritoneal cavity [6]. OC aggregates, mesothelial cells and surrounding blood cells all secrete cytokines that support OC survival in ascites so that both autocrine and paracrine mechanisms sustain OC EMT in ascites fluid and promote establishment of metastatic disease. The reverse process of MET results from loss of transient E-cadherin expression so that established metastatic OCs are frequently devoid of E-cadherin [7]. Pathways promoting EMT in OSE rely on complex interactions between OSE and its extracellular components and consist of autocrine and paracrine interactions with hormones and cytokines including transforming growth factor-β [8], epidermal growth factor [9], hepatocyte growth factor [10], endothelin-1 [11] and bone morphogenic protein 4 [12].
IL-1β and IL-6 have been found to induce EMT changes in a variety of cell types. Non-small cell lung cancer cells treated with IL-1β underwent EMT [13]. IL-1β enhanced EMT in bronchial epithelial cells and alveolar epithelial carcinoma cells through TGF-β1 [14, 15]. IL-1β induced EMT through upregulation of Zeb1 in colon cancer cells [16]; and promoted EMT in head and neck squamous cell carcinoma through Zeb1 and Snail [17, 18]. IL-6 promoted EMT changes in breast cancer by upregulating the expression of Twist [19]. Hepatocellular carcinoma mesenchymal tumors and mesenchymal cell lines were associated with increased IL-6 expression and knockdown of IL-6 in cell lines resulted in increased E-cadherin expression and increased chemosensitivity [20]. IL-6 induction of EMT was mediated by STAT3 in cervical and lung cancers [21-23]. Interestingly, epidermal growth factor/epidermal growth factor receptor signaling induces EMT in cultures of OC cell lines and is also associated with enhanced expression and secretion of IL-6, resulting in increased cellular motility [24].

In addition to promoting a migratory and invasive phenotype, EMT may contribute to patient mortality by: conferring paclitaxel resistance in epithelial OC cells [25]; inducing myocyte differentiation into CD14+/KDR+ pro-angiogenic cells thereby promoting tumor angiogenesis and vascularization [26] and; inducing the differentiation of stromal mesenchymal stem cells into tumor associated fibroblasts that support disease progression through matrix remodeling [27]. In this last context, it is interesting that both in vivo and in vitro studies indicate that IL-6 produced by stromal tumor associated fibroblasts enhances OC cell proliferation through activated STAT3 signaling [27].

This study suggests consideration of unique treatment options for BRCA1 mutation carriers. Treatment options should address the premalignant differences of mutation carriers before OC development, such as a possible prophylactic regimen of nitric oxide-releasing
NSAIDs (NO-NSAIDs) and/or cytokine-suppressive anti-inflammatory drugs that may prove useful in reducing the risk of BRCA1 mutation-associated OC. Similarly, the chronic inflammatory microenvironment potentially associated with specific BRCA1 mutation may respond to anti-inflammatory drugs as seen in colorectal cancer where targeting inflammation modulated tumor biology and resulted in improvement in patient outcome [28]. Clinical studies have already reported that BRCA-associated OC tumors have a higher sensitivity to platinum-containing treatments in comparison to mutation-negative tumors [29-31]. Therefore, enhanced treatment options may consider the addition of anti-inflammatory treatments, such as IL-1β neutralizing agents, in BRCA1-associated OCs in conjunction with standard therapy.

Phase I and II clinical trials utilizing recombinant human IL-6 as adjuvant treatment in conjunction with chemotherapy increased OC patient platelet count alleviating thrombocytopenia [32, 33]. As stated earlier, IL-1 increases production of IL-6 [34, 35]; therefore, studies investigating the effect of IL-1 on the development of OC are clinically important. A better understanding of IL-1 cytokines’ functions in relationship to OC may help to identify novel prophylactic drug treatments and/or treatments after disease occurrence.

Another phase I clinical trial showed recombinant human IL-1α treatment having minor antitumor effect in recurrent OC [36]. Nonetheless, clinical trials showed that recombinant human IL-1α treatment accelerated platelet recovery during chemotherapy and reduced carboplatin-induced thrombocytopenia in patients with recurrent OC [37]; as well as in patients with ovarian and other cancer types (gastrointestinal, breast, melanoma, lung, head & neck, sarcoma, and prostate) who did not receive concomitant chemotherapy [38]. However, IL-6 is an effective hematopoietic growth factor and plays a direct role in megakaryocyte differentiation into platelets [32], and it may prove a better option than utilizing recombinant IL-1α.
In contrast, due to IL-1β’s cancer-promoting activities, inhibiting IL-1β function represents a promising new avenue for therapeutic intervention. Developing novel IL-1β antagonists that block IL-1β’s ability to bind to its receptor should suppress IL-1β’s VEGF-mediated angiogenesis, invasiveness, and chemoresistance. While published clinical trials of IL-1β as a therapeutic target in treatment of cancer are limited, a phase I trial showed that treatment with recombinant human IL-1β elevated platelet levels in patients with gastrointestinal cancer [39]. IL-1β’s ability to increase platelet levels may be partially due to inducing production of IL-6. However, due to the aggressiveness of OC and IL-1β’s promotion of cancer progression, it may prove more beneficial to evaluate IL-1β as a treatment target rather than as a therapeutic agent. Therefore, as a treatment target, effective agents would likely mimic the action of IL-1β’s natural antagonist, IL-1Ra, and/or to neutralize IL-1β. Currently, a recombinant IL-1Ra, Anakinra, which blocks IL-1β and IL-1α from binding to their receptors, is used for the treatment of rheumatoid arthritis [40]. Likewise, the fusion glycoprotein, Rilonacept, which works by binding to IL-1α and IL-1β with high affinity to neutralize both molecules is yet another anti-inflammatory therapy currently in use [40]. Lastly, Canakinumab, a fully humanized monoclonal antibody highly specific for IL-1β, is currently in clinical use to suppress inflammation [40]. Consequently, these anti-inflammatory medications may be valuable for their capability to reduce IL-1-induced disease processes that may initiate and promote OC.

Lastly, I began molecular, biochemical and structural characterization of the predicted BRCA1 185delAG truncated protein. My analyses predict that BRAT is a small, hydrophobic protein located in the nucleus with potential DNA activating and/or inactivating capabilities. This work has illuminated possible mechanisms of 185delAG-dependent activity in the cell. The production of a BRCA1 185delAG protein tool had not been accomplished previously, but is
now available and will allow for further protein characterization including ascertaining its 3D structure and functions in the cell. Taken together, developing tools for the functional analyses of the different BRCA1 mutations may improve our understanding of the heterogeneity and etiology of OC, the most lethal gynecologic cancer.

References


Kamisha Woolery completed her undergraduate degree studies at the University of South Florida, where she graduated with a B.S. degree in Biomedical Science. During this time she participated in a summer Interdisciplinary Perspective on Women’s Health Research Experience for Undergraduates through the University of South Florida, College of Medicine and the Department of Women Studies. For the following two years, she worked as a Research Assistant for Dr. Andreas Seyfang Ph.D. at the University of South Florida, College of Medicine in the Molecular Medicine department. Kamisha joined the Medical Science Ph.D. Program in the USF College of Medicine in 2009 and received a Master’s degree in Medical Science in 2011. In 2010, she was awarded the National Science Foundation Florida-Georgia Louis Stokes Alliance Minority Participation Bridge to the Doctorate award. She presented her research at the 2011 American Association of Cancer Research Annual Meeting in Orlando, FL; the Annual Biomedical Research Conference for Minority Students in St. Louis, MO in 2011; and at the USF Health Research day in 2011, where she was awarded Outstanding Poster Presentation.