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Original Article

Beyond heredity mutations: alterations in the Fanconi anemia/BRCA pathway and its role in ovarian tumorigenesis

Laura E Hays[1], Gabrielle Choonoo[2], Shannon McWeeney[2,3], Tanja Pejovic[1]

[1]Department of Obstetrics & Gynecology, and The Knight Cancer Institute, Oregon Health & Science University, Portland, Oregon; [2]OHSU Knight Cancer Institute Bioinformatics Shared Resource, Oregon Health & Science University, Portland, Oregon; [3]Division of Bioinformatics and Computational Biology, Department of Medical Informatics and Clinical Epidemiology, Oregon Health & Science University, Portland, Oregon

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Abstract: The Fanconi anemia (FA)/BRCA pathway consists of fifteen proteins that mediate DNA homologous recombination (HR) and promote chromosomal stability. Here we review the evidence that genetic and epigenetic alterations in *BRCA2* (*FANCD1*), *BRIP1* (*FANCJ*), *FANCD2*, *FANCF*, *PALB2 (FANCN*) and *RAD51C* (*FANCO*), render ovarian cells susceptible to malignant transformation. In addition, we discuss the paradoxical findings that *BRCA2* and *FANCD2* are upregulated in subsets of ovarian cancer patients and how these findings suggest potential tailored therapies.

Keywords: Fanconi anemia, DNA repair, ovarian cancer, FANCD2

Introduction

A growing body of evidence demonstrates that proteins in the Fanconi anemia/BRCA (FA/BRCA) DNA repair pathway play pivotal roles in ovarian cancer suppression. Fanconi anemia is an inherited bone marrow failure syndrome that is characterized by chromosomal instability and hypersensitivity to DNA cross-linking agents, including cisplatin. In fact, increased chromosomal breakage in response to DNA cross-linkers (mitomycin C and diepoxybutane) is the accepted diagnostic test for FA. In addition to anemia, FA patients are highly predisposed to leukemia, head and neck squamous cell carcinoma, and gynecological cancers (cervical and vulvar) [1].

FA is caused by homozygous or X chromosome-linked deletion of one of 15 different genes, eight of whose gene products (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM) form a nuclear core complex that facilitates ubiquitination of FANCD2 and FANCDI. The ubiquitinated FANCD2/FANCI heterodimer then functionally interacts with down-stream FA proteins (FANCD1/BRCA2, FANCJ/BRIP1, FANCN/PALB2, FANCO/RAD51C, and FANCP/SLX4) to mediate DNA damage responses. The role of these proteins in promotion of chromosomal stability by mediating repair of DNA cross-links and double-strand breaks has been well established. In fact, FA proteins functionally interact with many members of the DNA homologous recombination (HR) pathway, including BRCA1. Although HR proteins and others interact with FA proteins, only genes that have been demonstrated to cause Fanconi anemia are designated as FANC genes and are the focus of this review (**Table 1**).

Although FA proteins play an essential role in suppressing chromosomal instability, there is substantial evidence that these proteins are multi-functional. Specifically, FA proteins suppress hyper-responsiveness to apoptotic cues [2-4], over-production of inflammatory cytokines [3, 5-7] and overproduction of reactive oxygen species [8, 9]. The potential contribution of these other functions of FA proteins to ovarian malignant transformation remains unclear. However, a recent report demonstrat-

Table 1. FA Genes and Relationship to Ovarian Cancer

FA Gene	Other Names	Function*	Association with Ovarian Cancer
FANCA	–	Core	–
FANCB	–	Core	–
FANCC	–	Core	–
FANCD1	BRCA2	Downstream	Hereditary mutations and epigenetic misregulation
FANCD2	–	Downstream	Decreased expression in HR women and overexpression in sporadic cases with poor prognosis
FANCE	–	Core	–
FANCF	–	Core	Hypermethylation in sporadic OvCa
FANCG	–	Core	–
FANCI	–	Downstream	–
FANCJ	BRIP1/BACH1	Downstream	Hereditary mutations
FANCL	–	Core	–
FANCM	–	Core	–
FANCN	PALB2	Downstream	Hereditary mutations and hypermethylation (?)
FANCO	RAD51C	Downstream	Hereditary mutations
FANCP	SLX4	Downstream	–

*For simplicity, function is defined here as member of the nuclear core complex (core) or functioning downstream of the core complex (downstream).

ed that loss of cytoplasmic FANCD2 staining in breast cancers strongly correlated with poor prognosis, suggesting an important non-nuclear function of FANCD2 in breast cancer suppression [10].

In 2002, the *FANCD1* gene was identified to be *BRCA2*, emphasizing the importance of this pathway in suppression of ovarian tumorigenesis. However, there is no increased incidence of ovarian cancer in [1] FA patients which may in part be due to early mortality (median age of survival is 14-25 years) from other pathologies (bone marrow failure or squamous cell malignancies) or [2] in parents of FA patients. This may be accounted for by the fact that the vast majority of FA is caused by mutations in three FA genes (*FANCA, FANCC,* and *FANCG*) that have not been implicated in ovarian cancer. Although, one case of breast cancer in an *FANCA* patient and a small increased susceptibility of *FANCC* carriers to breast cancer has been reported. Subsequent work by different groups has identified, however, that at least three of the less common FA genes are involved in hereditary ovarian cancer susceptibility (*FANCJ/BRIP1, FANCN/PALB2*, and *FANCO/RAD51C*). Other evidence suggests that epigenetic changes in and altered expression of FA genes contributes to both sporadic and hereditary ovarian cancer. This review will detail the evidence supporting the important role of the FA/BRCA pathway members in both suppression and promotion of ovarian cancer and how these FA/BRCA alterations may direct tailored treatments.

Hereditary mutations in ovarian cancer

It has been reported that approximately 10% of ovarian cancers are believed to be hereditary and the vast majority of these cases have been attributed to mutations in the *BRCA1* and *BRCA2* genes. Bi-allelic inactivation of *BRCA2* (*FANCD1*) causes Fanconi anemia, with a particularly severe phenotype that includes brain tumors. Recently other hereditary mutations have been found that confer ovarian cancer susceptibility and many of which are in the FA pathway, including *FANCJ* (*BRIP1/BACH1*), *FANCN* (*PALB2*), and *FANCO* (*RAD51C*). Although the relative proportion of these mutations in hereditary ovarian cancer and the total number of women with hereditary ovarian cancer was believed to be small, Walsh recently reported that the overall percentage of women with hereditary mutations was higher than thought (24%) and many women with hereditary mutations present without a family history (> 30%) [11]. Furthermore, they detected by parallel sequencing in a method they named 'BROCA' that 6% of all ovarian cancers tested had mutations in other genes, with 2% having mutations in non-*BRCA2* FA genes (*FANCJ, FANCN, and FANCO*). The contribution of FA genes to ovarian tumorigenesis may in fact be higher as [1] BROCA testing does not include other FA genes (although mutations in other FA genes may be exceedingly small as FA parents are not predisposed to breast or ovarian cancer, as described above) and [2] as will be discussed below non-genetic alterations in the FA path-

way may play an important role in ovarian tumorigenesis. As the evidence and connection of the FA proteins to hereditary ovarian cancer was comprehensively reviewed by Pennington and Swisher [12] in this review will focus on evidence for non-genetic alterations in the FA pathway that contribute to both sporadic and hereditary ovarian cancer.

Epigenetic regulation of FA pathway members

FANCF methylation

A possible role of altered FANCF expression in ovarian cancer, interestingly the only core complex FA protein so far implicated in the etiology of ovarian cancer, was first reported in 2003 [13]. Taniguchi demonstrated that two ovarian cancer cell lines were deficient for mono-ubiquitinated FANCD2 due to methylation-mediated loss of *FANCF* expression. Furthermore they found that 21% (4/19) of primary ovarian tumors from patients with no history of breast or ovarian cancer displayed *FANCF* methylation, while corresponding peripheral blood samples did not. Subsequent reports by other groups have varied in the relative proportion of *FANCF* methylation positive ovarian cancer samples. Specifically, two groups found, respectively, that 27.8% (5/18) [14] and 13.2 (7/53) [15] of primary ovarian tumors possessed *FANCF* methylation, while in contrast two others found either no (0/106) (16) or low (2.2%; 3/143) [17] levels of methylation. Differences may be due to stage, histological subtype, or treatment history as exemplified by Swisher who showed that a small but detectable fraction (3%; 3/93) of primary tumors (pre-treatment) displayed methylation (3/93), but no tumors from post-treatment or recurrent patients displayed methylation [17]. Alternatively, differences may be due to ethnic compositions of tumor samples from where they were collected, i.e. United States [13, 17], China [14], and United Kingdom [15, 16]. Regardless, the preponderance of evidence suggests that a small, but not insignificant proportion, of sporadic ovarian cancers are positive for *FANCF* methylation, which may have important considerations for treatment as discussed later in the review.

Mice deficient for the murine homolog of FANCF (Fancf) display small ovaries with reduced follicles and spontaneously develop ovarian tumors, demonstrating a critical function for FANCF in ovarian function [18]. The tumors from these mice are granulosa cell tumors and luteomas, which correlates with another study that shows that 24% (6/25) of human ovarian granulosa cell tumors are methylated at *FANCF* [19]. Therefore, aberrant FANCF function may play a critical role in both epithelial and non-epithelial ovarian cancers.

Methylation of other FA genes (FANCN/PALB2 and RAD51C)

A few reports have suggested that methylation of other FA genes may contribute to ovarian tumorigenesis. Potapova et al found that 7.5% (4/53) of sporadic ovarian tumors and 0% (0/9) of inherited tumors contained methylation at the *FANCN/PALB2* gene [20]. Although the numbers are small, this report suggests that analogous to *BRCA1* and *BRCA2*, alterations in expression of *FANCN/PALB2* may contribute to both inherited and sporadic ovarian cancer. In addition, one recent report detected *RAD51C* promoter methylation in 2.5% (1/39) of hereditary ovarian cancer patients without BRCA1/2 mutations. The numbers of tumors tested in both of these studies were small, demonstrating the need for further studies for confirmation. It should be noted that methylation of individual FA genes in ovarian cancer may be small, but together may add up to a significant proportion and as alterations in these different genes renders cells susceptible to the same molecular agents (i.e. platinum agents and PARP inhibitors), implications for putative treatments would be the same.

The use of murine models to clarify the roles of *Fancn* and *Fanco* in ovarian tumorigenesis are limited by the fact that whole animal knockouts of these genes are embryonic lethal, analogous to *Brca1* and *Brca2*.

Other alterations in the FA pathway

In addition to epigenetic changes, several groups have reported altered FA gene expression in ovarian cancer samples, although the putative molecular and genetic mechanisms underlying aberrant expression have not been delineated.

FANCD2

In work done by our group, expression of FANCD2 protein and mRNA was found to be

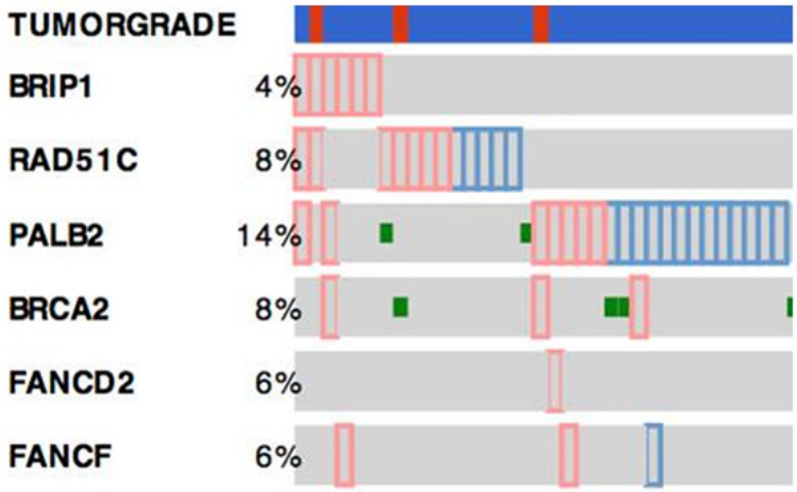

Figure 1. Oncoprint for the 6 selected genes in the Ovarian TCGA Data. This signatures is altered in 55 (35%) of cases. The OncoPrint visualization is from CBioPortal (http://www.cbioportal.org) with individual genes represented as rows, and individual cases as columns. Color coding and shapes allows multiple alterations in a subject's to be distinguished easily. Key for this figure: Tumor Grade color coding: Blue = G3; Red=G2; Alterations: Green = mutation, Red = mRNA up-regulation; Blue = mRNA down-regulation.

down-regulated in 60% (3/5) of pathologically normal ovaries from women with a family history of ovarian cancer (but no *BRCA1/2* mutations) and 12.5% (1/8) of women with ovarian cancer [21]. FANCD2 reduced expression was restricted to the ovaries as normal FANCD2 expression was detected in peripheral blood from these same patients. In the ovaries, no DNA mutations were detected in the *FANCD2* gene or its promoter nor was aberrant methylation detected in *FANCD2* or ten other FA genes screened. Although the numbers were small in the original study, we have found significant FANCD2 down-regulation in an expanded study (unpublished data). To further support a role for FANCD2 in ovarian tumor suppression, another group found that *FANCD2* mRNA expression was low in borderline and stage III ovarian tumor samples [22].

The mechanism underlying repressed FANCD2 expression remains enigmatic, but several possibilities include aberrant chromatin and miRNA deregulation. To support the latter, one group identified a novel genetic variant in the *miR-191* gene in an ovarian cancer family that increased its own expression. One of the putative targets of this miRNA is *FANCD2* and ex vivo experiments demonstrated miR-191 overexpression suppresses FANCD2 expression by approximately 40%. Further experiments are needed to identify the mechanisms involved in FANCD2 repression, but may be of particular interest as they could potentially function as targets for early therapeutic intervention to restore FANCD2 expression and prevent malignant transformation in high-risk women.

To support an important role for FANCD2 in ovarian development and malignant transformation, mice deficient for Fancd2 display underdeveloped ovarian follicles and the majority of mice develop epithelial ovarian tumors after 15 months of age. Murine models for Fancd2 and the other FA genes described above allow delineation of the contribution of these genes to the etiology of FArelated ovarian cancer and furthermore function as pre-clinical models for potential tailored therapies.

Loss of FA pathway and treatment

PARP inhibitors were proposed to be efficacious in cells deficient for BRCA1 and BRCA2 protein due to synthetic lethality and have shown promise in treatment of BRCA1/2-deficient breast cancer. The efficacy of these drugs in BRCA1/2-deficient ovarian cancers is still under investigation. Analogous to BRCA1/2-deficient cells, cells with deficiencies in FA genes (*FANCA* and *FANCD2*) are hypersensitive to PARP inhibitors. Therefore, cells with alterations in FA genes or FA gene expression, identified by BROCA testing or other means, may be sensitive to the same strategies proposed for BRCA1/2 positive tumors.

As loss of FA/BRCA pathway most likely promotes malignant transformation as an early event through genomic instability, strategies destined to either restore FA/BRCA expression or eliminate cells deficient for FA/BRCA genes before tumor formation present a tantalizing possibility. For example, as discussed above,

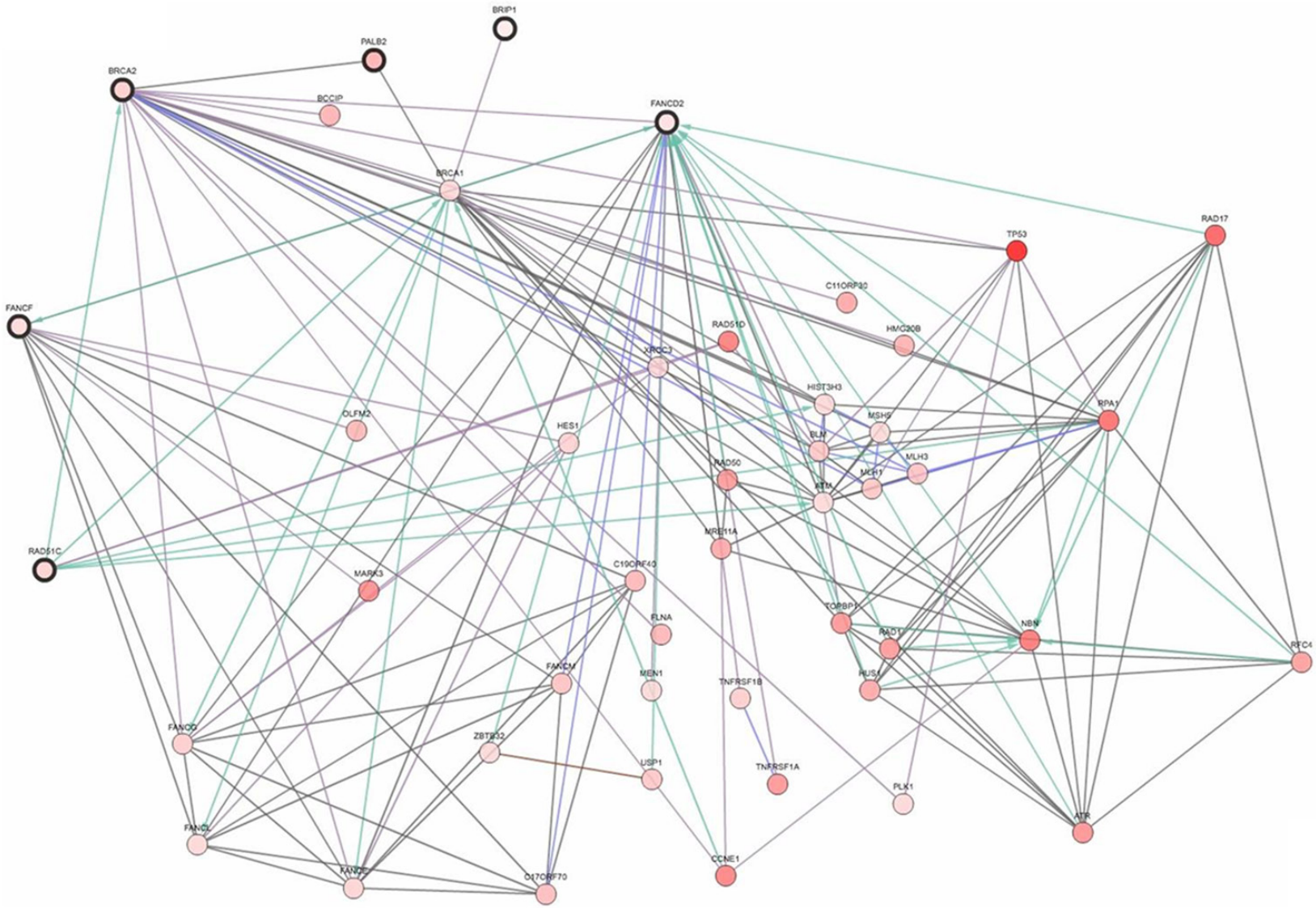

Figure 2. Network Visualization from the Ovarian TCGA data via CBioPortal (http://www.cbioportal.org). Circles represent genes and lines connecting the genes indicate interactions. The 6 selected genes ("seeds" for the network) are indicated by bold black lines around the circles. Pathway and interaction network data to generate the figure are are provided from Pathway Commons (http://www.pathwaycommons.org). Genes in the network were filtered to only include neighbor genes with a frequency of alteration of 6% or higher in the TCGA data set.

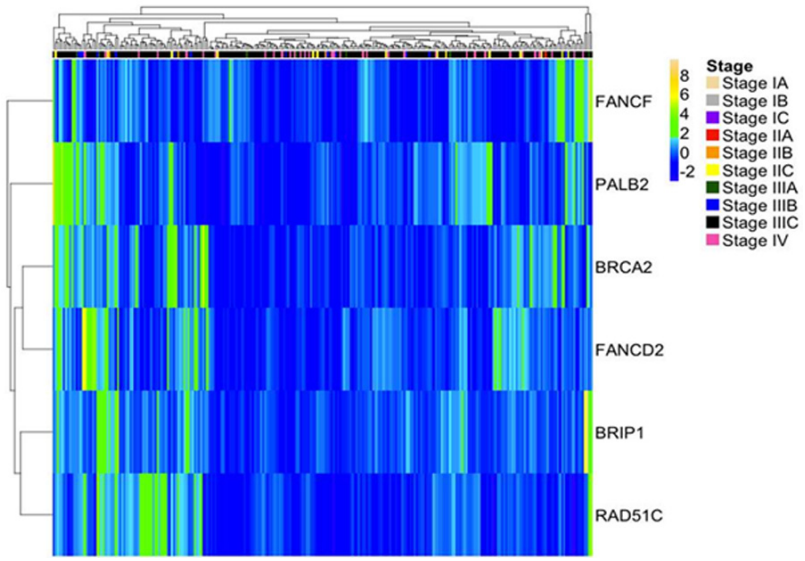

Figure 3. Heatmap of RNA-seq expression for the TCGA ovarian cancer patient samples (N=261) for the selected genes. Z-scores are utilized for the expression values. Color coding at the top indicates the Stage for each patient at the time the sample was taken. Data was queried from cBioPortal via the CGDS-R package, which is a R-Based API for accessing the MSKCC Cancer Genomics Data Server (CGDS).

FANCD2 expression was found to be low in a majority of non-cancerous ovaries (but not peripheral blood) from high-risk women. If the mechanism of FANCD2 repression could be identified, therapies that would restore FANCD2 repression may be developed to potentially inhibit malignant transformation in these women.

Paradoxical upregulation of FA/BRCA pathway in sporadic ovarian cancer

It is clear from the evidence cited above that many of the FA/BRCA proteins function as tumor suppressors, as reduced expression of these genes either through DNA mutations, promoter methylation, or other unknown molecular alterations correlate with ovarian cancer susceptibility and furthermore murine models of many of the genes spontaneously develop ovarian cancer. However, paradoxically, overexpression of these genes may also promote ovarian tumorigenesis as upregulation of these genes has been detected in ovarian tumors. Specifically, *BRCA2* mRNA was found to be over-expressed in tumor tissue vs. non-tumor tissue in sporadic ovarian cancer [23] and high expression of FANCD2 protein correlated with women with poor prognosis in our study [24].

The contribution of FA gene overexpression to ovarian cancer is unknown, however one likely possibility is that FA genes are upregulated after chemotherapy as a mechanism of carboplatin resistance. To support this, FANCD2 overexpression was highest in women with recurrent disease of less than one year. Regardless of the mechanism, upregulation of these genes and their protein products would most likely render these cancers resistant to both DNA cross-linkers and strategies that are dependent on loss of the FA/BRCA pathway (i.e. PARP inhibitors). It has been proposed by several groups that targeted inactivation of the FA/BRCA pathway with chemicals such as curcumin may be an effective strategy for sensitizing tumors to crossinkers. These tumors might be ideal candidates for these treatments.

Bioinformatics and tailored therapies

As discussed above, alterations in the FA/BRCA pathway may make ovarian cancer cells more sensitive to specific therapies. Therefore, identification of alterations in these pathways in both hereditary and sporadic tumors is an important goal. Currently, this can be accomplished by *BRCA1/2* sequencing at Myriad Genetics, BROCA sequencing at the University of Washington, and different other gene panels by several companies. However, molecular and epigenetic alterations that render ovarian cancer cells sensitive or resistant to FA/BRCA specific treatments would most likely not be detected by traditional DNA sequencing. We have performed a comprehensive analysis of publically available TCGA ovarian cancer data set for the six FA genes of interest (**Figures 1**-**3**) including DNA mutation and mRNA expression status and most frequently altered gene network. This analysis reveled the alteration in over 35% of ovarian cancer cases. Therefore, this and other computational approaches including gene and

miRNA expression measurements via microarrays or next generation sequencing will be successful in identifying other women that would be responsive to these therapies.

Summary

Inherited or acquired defects in DNA repair may be predictive of ovarian cancer risk. On the other hand, the significance of endogenous DNA repair pathways in ovarian cancer has been well established. Detecting defects in DNA repair has prognostic value: tumors that have a diminished capacity for DNA repair may be more susceptible to platinum-based therapies. The ability to modulate DNA repair pathways may therefore be of significant clinical value in cancer prevention via restoration of these pathways prior to transformation, and in patient survival, via disruption of these pathways in tumors. The Fanconi anemia (FA)/BRCA pathway is a key pathway employed to repair DNA crosslinks and hence may be a promising target for both ovarian cancer prevention and treatment.

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Address correspondence to: Tanja Pejovic, Department of Obstetrics & Gynecology, and The Knight Cancer Institute, Oregon Health & Science University, Portland, Oregon. E-mail: pejovict@ohsu.edu

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