

Fanconi Anemia Signaling and Biological Information Flow

Kanani Hokutan¹ and Peiwen Fei^{2*}

¹University of Hawaii, Honolulu, Hawaii, USA.

²University of Hawaii Cancer Center, Honolulu, Hawaii, USA.

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ABSTRACT

In 1957, Francis Crick explained biological information flows from DNA to RNA to protein. Advances in biological studies reveal this can be modified to DNA, epigenetic, RNA, protein and metabolite. Fanconi Anemia (FA) signaling mainly repairs DNA damage and thus plays a pivotal role in maintaining the integrity of the genome. Impaired FA signaling is involved in all steps of the biological information flow, contributing to the development of a variety of human disorders, especially aging and cancers. Here we discuss how FA signaling maintains biological information flow to protect human cells from going awry and developing into disease.

Keywords: Biological information Flow, Fanconi Anemia (FA) Signaling, Cancer development, Cancer treatment

Abbreviations: FA: Fanconi Anemia; ICL: Interstrand Crosslink; NER: Nucleotide Excision Repair; HR: Homologous Recombination; FAAP: FA Associated Protein; RPA: Replication Protein A; MMC: Mitomycin C; AML: Acute Myeloid Leukemia; TCGA: The Cancer Genome Atlas; HNSCC: Head And Neck Squamous Cell Carcinoma; CRC: Colorectal Cancer; MMR: Mismatch Repair; aa: Amino Acid; TNBC: Triple Negative Breast Cancer; ALL: Acute Lymphoblastic Leukemia; PAS: Polyadenylation Site; APA: Alternative Polyadenylation; miRNA: MicroRNA; NSCLC: nonsmall cell lung cancer; SCC: Squamous Cell Carcinoma; LOH: Loss Of Heterozygosity; MiTF: Microphthalmia-Associated Transcription Factor; TMZ: Temozolomide; TFG: Trk-Fused Gene; MS: Mass spectrometry; Me-D/Me-P: Methylated Distal/Methylated Proximal; PARPi: PARP inhibitor; BER: Base excision repair; SSB: Single strand break; DSB: Double Strand Break; CLL: Chronic Lymphocytic Leukemia; HGSOC: High-Grade Serous Ovarian Carcinoma; PDX: Patient-Derived Xenograft; DNMTi: DNMT Inhibitor

INTRODUCTION

The central dogma of molecular biology describes the transfer of information during DNA replication, transcription to RNA and translation to proteins [1,2]. However, Temin and Baltimore's discovery of reverse transcriptase in 1970 provided evidence that information can be transferred in the opposite direction, from RNA to DNA [1]. Years later we continue to discover regulatory features and processes that alter information dynamics [1]. Studies at the genomic, epigenomic, transcriptomic, proteomic and metabolomic levels have proven to interact but perhaps not in a unidirectional flow as once proposed [2].

The FA signaling pathway functions to repair DNA damage, specifically interstrand crosslinks (ICL), a lesion that blocks both DNA replication and transcription [3]. This signaling system detects and removes ICLs with other DNA repair mechanisms, including nucleotide excision repair (NER) and homologous recombination (HR) [4] in order to maintain genomic stability. FANCM, a DNA translocase, creates a

complex with MHF1 and MHF2 and recognizes the ICL as a result of replication fork stalling [3, 5]. The FANCM-MHF1-MHF2 complex and PCNA recruit the FA core complex and FANCD2 and FANCI to chromatin, respectively [5]. The FA core complex, an E3 ubiquitin ligase, is composed of eight FA proteins (FANCA, B, C, E, F, G, L, M) and FA associated proteins (FAAP100, FAAP20, and FAAP24) and in conjunction with UBE2T/FANCT, an E2 ubiquitin-conjugating enzyme, adds ubiquitin to targets FANCD2 on

Corresponding author: Peiwen Fei, MD PhD, University of Hawaii Cancer Center, 701 Ilalo Street, Honolulu, HI 96813, Tel: 808-356-5786; E-mail: pfei@cc.hawaii.edu

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Lys 561 and FANCI on Lys 523. The monoubiquitination of the ID2 complex serves as the activation step of FA signaling [3]. The ubiquitinated ID2 complex allows for SLX4/FANCP, a scaffolding protein, to recruit DNA endonucleases MUS81, SLX1 and XPF/ERCC4/FANCO, which generate a DNA adduct and a double-strand break. The double-strand break is removed by CtIP, MRN, and EXO1, which creates a 3' single-stranded DNA overhang that is coated by replication protein A (RPA). RAD51/FANCR recognizes the RPA-covered single-stranded DNA and leads to the formation of a recombination filament assisted by BRCA2/FANCD2, FANCN/PALB2, RAD51C/FANCO, BRIP1/BACH1/FANCI, and XRCC1/FANCU. The recombination filament searches for a homologous double-stranded DNA to complete the process [5]. FA pathway alterations have major consequences, leading to disease. Biallelic germline mutations in FA genes

results in Fanconi anemia (FA), a rare genetic syndrome presenting with bone marrow failure, congenital defects, and hypersensitivity to DNA damaging agents such as cisplatin, mitomycin C (MMC), and diepoxybutane [4,6]. FA is a DNA repair disorder. Patients have increased levels of oxidative DNA damage, which causes inflammation and contributes to premature aging [7]. FA patients are also predisposed to many cancers. The most common are acute myeloid leukemia (AML) at a 700-fold higher incidence than the normal population and solid tumors such as head and neck at a 50-fold higher incidence [8]. An impaired FA pathway is also found in the normal population and likewise associated with cancer. There are 22 known FA genes (FANCA/B/C/D1/D2/E/F/G/I/J/L/M/N/O/P/Q/R/S/T/U/V/W) (Table 1).

Table 1. FA complementation groups. Summary of 22 FA complementation groups with corresponding synonyms and chromosome location.

Symbol	Name	Synonyms	Chromosomal location
FANCA	FA complementation group A	FACA, FANCH, FAA, FA-H, FAH, FA1, FAA, FA	16q24.3
FANCB	FA complementation group B	FAB, FAAP95, FA2, FAAP90, FACB	Xp22.2
FANCC	FA complementation group C	FACC, FAC, FA3	9q22.32
BRCA2	BRCA2 DNA repair associated	FANCD1, FACD, FANCD, FAD, FAD1, BRCC2, XRCC11, GLM3, PNCA2, BROVCA2	13q12.13
FANCD2	FA complementation group D2	FACD, FANCD, FAD, FA-D2, FA4	3p25.3
FANCE	FA complementation group E	FAE, FACE	6p21.31
FANCF	FA complementation group F	FAF	11p14.3
FANCG	FA complementation group G	FAG, XRCC9	9p13.3
FANCI	FA complementation group I	KIAA1794	15q26.1
BRIP1	BRCA1 interacting protein C-terminal helicase 1	FANCI, BACH1, OF	17q23.2
FANCL	FA complementation group L	FAAP43, PHF9, POG	2p16.1
FANCM	FA complementation group M	POF15, SPGF28, FAAP250, KIAA1596	14q21.2
PALB2	Partner and localizer of BRCA2	FANCN, FLJ21816, PNCA3	16p12.2
RAD51C	RAD51 paralog C	FANCO, RAD51L2,	17q22

		BROVCA3, R51H3	
SLX4	SLX4 structure-specific endonuclease subunit	FANCP, BTBD12, KIAA1987, MUS312	16p13.3
ERCC4	ERCC excision repair 4, endonuclease catalytic subunit	FANCO, RAD1, XPF, XFEPS, ERCC11	16p13.12
RAD51	RAD51 recombinase	FANCR, RAD51A, RECA, HRAD51, HsRad51, HsT16930, BRCC5, MRMV2A	15q15.1
BRCA1	BRCA1 DNA repair associated	FANCS, RNF53, BRCC1, PPP1R53, BRCA1, BROVCA1, IRIS, PNCA4, PSCP	17q21.31
UBE2T	Ubiquitin conjugating enzyme E2 T	FANCT, PIG50, HSPC150	1q32.1
XRCC2	X-ray repair cross complementing 2	FANCU	7q36.1
MAD2L2	mitotic arrest deficient 2 like 2	FANCV, MAD2B, REV7, POLZ2	1p36.22
RFWD3	ring finger WD repeat domain 3	FANCW, RNF201	16q23.1

Researchers continue to identify new FA genes. The most recently identified is FANCW. The E3 ubiquitin ligase RFWD3 functions in HR. RPA is polyubiquitinated by RFWD3 in response to DNA damage, which is essential for proper HR [9]. A compound heterozygous mutation in the RFWD3 gene on chromosome 16q23 led researchers to identify FANCW. The following mutations, c.205_206dupCC and c.1916T>A, were found in a 12 year old German girl with FA. Patient cells showed an increase in chromosomal breakage, reduced survival and cell cycle arrest in G₂ after exposure to MMC and other DNA damaging agents. In vitro studies of the FANCW variant indicate less relocalization to the nucleus and chromatin, disrupted physical interaction with RPA proteins and impaired HR compared to wild-type RFWD3 [10]. Many have performed studies to determine the role of FA signaling in cancer. In this review, we focus on FA signaling in cancer and highlight its influences at all levels of biological information flow.

DNA LEVEL: FA GENE MUTATION AND CANCER

Monoallelic mutations in FA genes do not result in the FA phenotype; however, in line with FA patients, these individuals are also susceptible to multiple cancers [4, 6, 11]. Germline monoallelic mutations in FA genes increase risk for breast, ovarian and pancreatic cancer [4]. Additionally, analysis from The Cancer Genome Atlas (TCGA) reports somatic FA gene alterations are common in malignancies [12, 13]. Genetic changes have consequences and the potential to alter epigenetics, RNA, protein and metabolites.

Next, we focus on FA pathway mutations at the DNA level found in human cancer.

Biallelic germline mutations in FA genes cause classical FA. Interestingly, researchers have shown biallelic mutations in FANCM do not develop FA and therefore may be mistakenly identified as a FA gene [14]. BRCA1/FANCS, BRCA2/FANCD1 and PALB2/FANCN gene mutations are associated with a high risk of breast cancer [14]. Previous studies show FANCM monoallelic mutations are low/moderate risk factors; however, data on biallelic mutation carriers were missing. Researchers found individuals with FANCM biallelic mutations are predisposed to cancer, display chemotherapy related hematological side effects, develop early menopause and are positive for chromosome fragility [14]. Their data show biallelic FANCM mutations have more evident clinical consequences than monoallelic carriers, and FANCM is not a canonical FA gene. But it appears to be a key FAAP owing to its roles in influencing ID2 monoubiquitination.

FANCM mutation studies have also been done in head and neck cancer. Researchers observed ICL-induced chromosomal breakage in nine of 17 head and neck squamous cell carcinoma (HNSCC) cell lines from patients without FA. They reported defective sister chromatid cohesion in five cell lines, and an inactivation of FANCM was responsible for chromosomal breakage [15]. Others identified gains on 16q23-24 are associated with FANCA amplification and correlates with reduced progression free survival after radiotherapy [16]. They silenced FANCA in

HNSCC cell lines with genomic gains on 16q23-24, which results in impaired clonogenic survival upon irradiation. When they overexpressed FANCA cell survival increased, DNA repair improved and chromosomal translocations were reduced. Sixty percent of patients have advanced stage HNSCC and are treated with cisplatin containing chemoradiation. Researchers performed a genome wide functional genetic screen to identify genes that influence the response to cisplatin in HNSCC cells [17]. Using siRNA, they identified the FA/BRCA pathway as the pathway for cisplatin response in HNSCC. Knockdown of BRCA1 and BRCA2 enhances cisplatin-induced cell death and SHFM1, a protein that interacts with BRCA2 also showed the same trend. Researchers found expression of these genes are upregulated in HNSCC cells compared to normal mucosa, and the expression profile of 84 genes predicts the prognosis of radiation and chemoradiation treated patients.

FANCA is the most frequently mutated of the FA genes [18]. In prostate cancer, germline mutation S1088F in FANCA in addition with FANCA loss of heterozygosity (LOH) are deleterious for FANCA function and contributes to patients' response to cisplatin [19]. The FANCA mutant protein disrupts FA core complex formation, enhances sensitivity to cisplatin and MMC and displays lower levels of ubiquitinated FANCD2 after treatment compared to wild type. When injecting mice with cells harboring the FANCA S1088F variant followed by Olaparib treatment, data showed tumors were more sensitive to Olaparib compared to vehicle treated. Also, in prostate cancer, the IMPACT study involves patients with BRCA1 and BRCA2 mutations. Their interim analysis shows men with BRCA2 mutations have an increased cancer incidence rate, diagnosed with disease at a younger age, and BRCA2 carriers were diagnosed with intermediate or high-risk prostate cancer [20].

FA genes have also been shown to participate in colorectal cancer (CRC). Researchers screened 206 patients with CRC for germline mutations in DNA mismatch repair (MMR) genes, MLH1 and MSH2, and FA genes and showed FA genes are silenced during disease progression and metastasis formation, while MLH1 and MSH2 genes are inactivated earlier [21]. Another group performed germline DNA whole exome sequencing in order to find new candidate germline predisposition variants for CRC. They collected samples from 74 patients from unrelated Spanish families and found enrichment for variants in FA genes- BRCA2, BRIP1/FANCI, FANCC, FANCE, and REV3L/POLZ [22]. Similarly, a recent study shows an increase of potentially disruptive variants in NTHL1, BRCA2 and BRIP1/FANCI in CRC [23].

Genomic studies have shown FA gene mutations are present in a variety of cancers (**Table 2**). Some alterations are associated with an increase in cancer risk and disease progression, which potentially disrupt other levels

- Including whether a base is methylated or not
- Varying splice sites and miRNA binding
- Mutating the amino acid (aa) sequence leading to changes in protein function and interaction and lastly,
- Altering metabolites. Data from sequencing technologies give researchers the ability to find new targets or validate targets proven to be risk factors for malignancy. Studies looking at specific genetic alterations and response to therapy provide clinicians with the knowledge to choose the best treatment options for their patients.

Table 2. FA gene mutation and cancer. Mutated FA genes found in associated cancer types.

Cancer Type	Symbol		
AML	FANCA	BRAC2	FANCD2
	FANCG		
Bladder	FANCG		
Breast	FANCB	FANCC	BRAC2
	FANCD2	FANCF	FANCG
	FANCI	BRIP1	FANCM
	PALB2	RAD51C	SLX4
	ERCC4	RAD51	BRCA1
	UBE2T	XRCC2	
Cervical	FANCA	FANCC	FANCF
	FANCL		
Colorectal	FANCC	BRAC2	FANCE
	BRIP1	BRCA1	
Esophageal	FANCD2	FANCE	FANCL
Gastric	FANCE	FANCF	
Head and Neck	FANCA	BRAC2	FANCD2

	FANCF	FANCM	BRCA1
Leukemia	FANCB	FANCE	FANCG
	FANCL	FANCM	ERCC4
Lung	FANCF		
Oral	FANCA	FANCC	FANCD2
	FANCF	FANCG	
Ovarian	FANCD2	FANCF	FANCG
	FANCM	PALB2	RAD51
	BRCA1	RFWD3	
Pancreatic	FANCA	FANCC	FANCG
	FANCM	PALB2	
Prostate	FANCA	BRAC2	FANCF
	FANCG	FANCI	PALB2
	BRCA1		
Testicular	FANCD2	BRIP1	FANCL
	RAD51C		

EPIGENETIC LEVEL: EPIGENETIC ALTERATIONS OF FA GENES FOUND IN CANCER

Building on information gained from genetic studies, studying the FA pathway at the epigenetic level allows for a deeper understanding of how these genes are associated with cancer. Two methylation patterns known to occur in cancer cells are wide areas of hypomethylation and hypermethylation at CpG islands in gene promoters [24]. Hypomethylation of proto-oncogenes leading to gene activation or hypermethylation of tumor suppressor genes resulting in gene silencing have the potential to alter gene activity. Many are interested in studying DNA methylation as findings can be used to predict outcomes and treatment efficacy [24,25]. Next, we highlight inactivation of FA gene promoters by methylation found in cancer.

One of the first to study FA gene methylation status and its association in cancer was D'andrea and colleagues in 2003. They showed methylation of BRCA1 promoter in 5-15% of nonfamilial ovarian cancer cases and 11-31% in nonfamilial breast cancers. Additionally, the FANCF promoter is hypermethylated in 20% of primary ovarian cancers. They found methylation and silencing of FANCF results in cisplatin sensitivity and demethylation in FANCF gene leads to acquired cisplatin resistance [26]. Their findings led other groups to perform follow up studies on FANCF methylation in ovarian cancer.

Researchers noted promoter methylation was higher in more advanced stage disease and reported a comparable hypermethylated FANCF promoter frequency, at 24% [27]. However, others showed only one of nine cell lines and 13.2% of samples were hypermethylated at the FANCF promoter in advanced ovarian cancer patients on a cisplatin based chemotherapy trial [28]. Likewise, another group looked at promoter methylation of BRCA1, MLH1 and

FANCF and if methylation influences chemotherapy response or alters protein expression after chemotherapy. Similar frequencies of BRCA1, MLH1, and FANCF promoter methylation occurred in primary carcinomas without previous chemotherapy, after neoadjuvant chemotherapy or in recurrent neoplasms. However, they reported low BRCA1 expression is associated with prolonged survival, and recurrent ovarian carcinomas have increased BRCA1 and/or BRCA2 protein expression after chemotherapy exposure [29].

FANCF promoter methylation has also been evaluated in other cancers such as cervical and breast. One group showed FANCF and BRCA1 are hypermethylated and FANCF expression is down-regulated in most cervical cancer cell lines. Additionally, inhibition of DNA methylation and histone deacetylases induces FANCF gene re-expression in cell lines [30]. In breast cancer, researchers evaluated ER α , BRCA1 and FANCF promoter methylation and showed CpG islands of ER α are methylated in 49.2% of primary breast cancers and are associated with high tumor grade and tumor subtype. However, they found FANCF methylation to be a rare event at 0.8% [31]. Similarly, a group from Japan also reported a low incidence of methylation of FANCF promoter region at 4% and no correlation was found between FANCF methylation and expression of ER, PR and HER2 in triple negative breast cancer (TNBC) [32]. However, BRCA1 promoter methylation was found in 16% of TNBC and is associated with lymphoid vessel invasion, high nuclear grade, low BRCA1 mRNA expression, loss of BRCA1 protein expression and shorter overall survival [33].

FA gene promoter methylation is also infrequent in bladder cancer and leukemia. It was shown FANCF promoter hypermethylation is rare in bladder cancer [34]. Additionally, the same group also examined FANCC and FANCG and were unable to detect mutations in either genes

in 23 bladder carcinoma cell lines and ten tumor tissues. They hypothesized alterations in these FA genes may occur as bladder carcinomas have large deletions of chromosomes 9p and/or 9q. In leukemia, FANCC and FANCL promoter methylation were analyzed in 143 AML and 97 acute lymphoblastic leukemia (ALL) samples. FANCC promoter methylation was found in one AML sample and three ALL samples. FANCL promoter was methylated in a single ALL sample. Although hypermethylation of the FA genes tested occurs infrequently, they were able to show the hypermethylated samples were more sensitive towards MMC in a colony formation assay [35].

Epigenetic alterations disrupt gene expression and have the potential to change proteins and metabolites. These lead to changes in the microenvironment that alter cell functions. Silencing of tumor suppressors promotes errors in DNA repair and ultimately leads to tumorigenesis [36]. Studying the FA pathway at the epigenetic level has provided beneficial information in regards to cancer treatment. Methylation studies of FA gene promoters identify high-risk patients and predict treatment efficacy and outcomes. However, most studies have looked at FANCF, leaving much of the FA pathway or signaling network unknown regarding epigenetic inactivation (Table 3) [36-40].

Table 3. FA gene promoter methylation and cancer. Methylated FA gene promoters in associated cancer

Symbol	Cancer
FANCB	Head and Neck
BRCA2	NSCLC, Ovarian
FANCF	Ovarian, Breast, Cervical, Head and Neck, NSCLC, Leukemia
PALB2	Breast, NSCLC
BRCA1	Ovarian, Breast, Gastric, NSCLC, Uterine, Bladder, CRC, Pancreatic

RNA LEVEL

Coding RNA variants and FA genes

Pre-mRNA splicing is an essential and regulated process for producing mature mRNA. RNA splicing follows 5' capping and 3' cleavage or polyadenylation [41,42]. Splicing is carried out by the spliceosome, which recognizes exonic and intronic boundaries in pre-mRNA thereby removing introns and joining exons [42,43]. Alternative splicing allows for the production of multiple mature mRNAs from one gene, and therefore increases protein diversity, interactions and signaling [44]. More than 50% of human genes have

alternative splice variants [45] and many cancer-related genes undergo alternative splicing or have transcriptional variants [42]. Here we recognize some of the FA genes with RNA variants. A summary can be found in (Table 4) [45-53].

Our lab identified a splice variant of FANCL, FAVL. FAVL encodes a protein with 272 aa, 258 aa which are identical to FANCL. This variant has 14 unique aa and joins exon 9 to exon 12, skipping exons 10 and 11. FAVL protein expression was increased in 50% of tissue samples tested from osteosarcoma and lung and prostate cancer [50]. Further, we showed FAVL expression is high in bladder cancer tissues and ectopic expression of FAVL in bladder cancer and normal cells resulted in an impaired FA pathway and MMC hypersensitivity. We performed in vitro and in vivo studies and showed high FAVL expression promotes cell growth and invasiveness and is associated with chromosomal instability [54].

The center player of the FA pathway, FANCD2 is another gene with transcriptional variants. Our lab recognized an overlooked FANCD2 variant, FANCD2-V2. We identify the long-known form as FANCD2-V1. Both variants share 1427 aa at their N-terminal and therefore share 95% sequence identity. The cDNA of FANCD2-V1 encodes 1451 aa whereas FANCD2-V2 encodes a 1471 aa [45]. FANCD2 has two polyadenylation sites (PAS), a proximal and distal. This allows for alternative polyadenylation (APA) to occur, which is an RNA processing mechanism that produces mRNA isoforms with different 3' UTRs [43]. Studying DNA methylation at the regions of the proximal and distal PAS revealed high DNA methylation at the proximal PAS results in proximal PAS usage and FANCD2-V2 production. Alternatively, if there is low DNA methylation at the proximal PAS, distal PAS is used and FANCD2-V1 is produced [55]. We further showed a high methylated distal or methylated proximal ratio (Me-D/Me-P) is associated with tumor tissues in multiple cancers, including lung, kidney, endometrial, colon and breast. Whether RNA splicing is directly involved in FANCD2 mRNA variants needs to be determined.

Another group identified three novel splice variants of RAD51C/FANCO mRNA in CRC tumors and cells. Exon 7 is removed from variant 1, exons 6 and 7 are removed from variant 2 and variant 3 excludes exons 7 and 8. They found variant 1 is over expressed in tumors and the RAD51C promoter is methylated in tumor cells [51]. The same group identified two forms of fusion mRNAs between RAD51C and ATXN7 in CRC tumors. Variant 1 was previously identified in MCF-7 breast cancer cell line and consists of RAD51C exons 1-7 and ATXN7 exons 6-13 and produces a truncated protein. Variant 2 is a fusion of RAD51C exons 1-6 and ATXN7 exons 6-13 and produces a 110 kDa protein which results in an impaired FA pathway [56].

Table 4. FA RNA variants and cancer. Common FA RNA variants found in cancer.

Symbol	Variant	Cancer
FANCA	FANCAins10A, FANCAΔ11, FANCAΔ30 and FANCAΔ31	Non-BRCA1/2 Breast Cancer
FANCC	FANCCΔ7	Leukemia, Ovarian, Breast
FANCD2	FANCD2-V1 FANCD2-V2	High grade tumors Normal, Low grade tumors
FANCE	FANCEΔ4	High risk non-BRCA1/2 Breast Cancer
FANCL	FAVL-skip exon 10 and 11	Bladder, Lung, Prostate, Osteosarcoma
RAD51C	Variant 1-skip exon 7 Variant 2-skip exon 6 and 7 Variant 3-skip exon 7 and 8 RAD51CΔ5 RAD51CΔ8	CRC Hereditary Breast and Ovarian Cancer
BRCA1	BRCA1-Δ11q BRCA1Δ15 BRCA1Δ15q	Human cancer cell lines Hereditary Breast and Ovarian Cancer
BRCA2	BRCA2Δ2 BRCA2Δ3 BRCA2Δ5 BRCA2Δ17 BRCA2Δ25	Hereditary Breast and Ovarian Cancer

Non-coding RNA-miRNA and FA genes

MicroRNAs (miRNAs) are small non-coding RNAs, which regulate gene expression. It has been shown that miRNAs can function as oncogenes or tumor suppressors, and dysregulated miRNA expression can be found in cancer [57]. Changes at the genetic level can alter miRNA binding. Perhaps losing binding where it once occurred or providing a target for miRNAs, which would not normally bind. The miRNAs are known to target FA pathway genes in tumor cells and we highlight those here.

One group found miR-503 expression is decreased in NSCLC tissues. miR-503 targets the 3' UTR of FANCA and results in decreased FANCA expression. Researchers suggest targeting FANCA may be a strategy for sensitization of NSCLC for cisplatin as these cells can be sensitized up to three fold when FANCA is inactivated [58]. Also, in NSCLC, others conducted a meta-analysis using potential target genes of miR-1, which are downregulated in human cancer. They reported miR-1 may be involved in the progression of lung SCC, a subtype of NSCLC, via the cell cycle, p53 signaling pathway, FA pathway and HR [59].

In myeloid malignancies, researchers detected impaired HR was linked to promoter methylation of BRCA1. BRCA1 knockdown increased sensitivity to PARP inhibition, and its expression is inversely related to miR-155 expression. Increase of miR-155 is associated with PU.1 and SHIP1

repression, which are myeloid differentiation factors [60]. These factors are found to be downregulated in leukemic transformation.

In a pre-leukemic mouse model, researchers identified miR-139-3p and miR-199a-3p were elevated in common myeloid progenitors from Ercc1-deficient mice compared to control. The miRNAs were also elevated in CD34+ bone marrow cells from FA patients. miR139-3p inhibits proliferation of myeloid progenitors; however, miR-199a-3p is an onco-miR and causes AML in their mouse model. miR-199a-3p targets include tumor suppressors PRDX6, RUNX1, and SUZ12 [61].

RNA studies provide another level of intricacy to study cancer. Aberrant splicing can cause many changes including, LOH in tumor suppressors [62] and variants translating into protein isoforms that promote growth and survival [63]. Other areas affected include apoptosis, the cell cycle, invasion, metastasis, angiogenesis and metabolism [63]. Analysis of alternative splicing changes in cancer revealed protein domain families are frequently mutated in tumors and disrupt protein-protein interactions in cancer pathways [64]. miRNAs targeting tumor suppressor FA pathway genes down regulate their expression and promote tumorigenic functions. We have learned changes in pre-mRNA splicing and miRNA are found in the FA pathway. Although this poses an even greater challenge, RNA variants and miRNAs

have therapeutic potential for cancer diagnosis and prognosis and provide researchers with targets [44,64].

PROTEIN LEVEL

FA protein and cancer

Studies have shown the FA pathway is highly expressed in cancers and therefore may serve as a therapeutic target. In metastatic melanoma with microphthalmia-associated transcription factor (Mitf), FA pathway proteins are over expressed. Down regulation of Mitf lowers protein expression of FA genes and silencing the FA pathway alters proliferation, migration and senescence, displaying this pathway as a therapeutic target for melanoma treatment [65]. In glioblastoma, FANCD2 protein expression is increased and associated with tumor grade. Primary cultures from grade III and IV gliomas express FANCD2 and when treated with Temozolomide (TMZ), FANCD2 is monoubiquitinated and produces nuclear foci suggesting an active FA pathway. Treatment of U87 cells, a glioma cell line with an intact FA pathway, with three FA pathway inhibitors-curcumin, EF-24 and DDN inhibited monoubiquitination after TMZ. Results were replicated in primary glioma cultures. In U138 cells, a glioma cell line with an impaired FA pathway, endogenous FANCD2 was reduced, and FA pathway activation was abolished after TMZ [66]. Data shows inhibiting the FA pathway in glioblastoma sensitizes cells after chemotherapy.

Patients often become resistant to chemotherapy. In multiple myeloma, NF- κ B subunits, RelB/p50 activate the FA pathway leading to FANCD2 expression and melphalan resistance. Researchers found using siRNA to block NF- κ B or bortezomib, a proteasome inhibitor, FANCD2 protein expression decreased, leading to melphalan sensitivity [67]. The standard treatment for patients with lung squamous cell carcinoma is a combination of platinum and gemcitabine; however, gemcitabine resistance determines treatment success. Researchers showed suppression of the FA pathway increased sensitivity of two lung squamous cell carcinoma (SCC) cell lines SK-MES-1 and KLN205 to gemcitabine. They found the CHK1 and FA pathways are compensatory in the repair of DNA damage. FANCD2 depletion in combination with a CHK1 inhibitor, MK-8776, potentiated cytotoxicity of gemcitabine; therefore, both CHK1 and FA pathways provide targets for the improvement of lung SCC therapy [68].

FA protein variants

Other groups have focused on FA protein variants and cancer risk association. One group examined the role of protein truncating variants in BRCA1 interacting protein C-terminal helices, BRIP1 (FANCD2) and breast cancer risk. They evaluated truncating variant p. Arg798Ter and 10 missense variants of BRIP1 in 48,144 cases and 43,607 controls of European origin (41 studies in the Breast Cancer Association Consortium). They further sequenced BRIP1 in

13,213 cases and 5,242 controls from UK, 1,313 cases and 1,123 controls from studies part of the breast cancer family registry and 1,853 familial cases and 2001 controls from Australia. Very few cases and controls from the European samples and sequencing studies were found, suggesting the p.Arg798Ter variant is not associated with an increase in breast cancer risk [69]. In another large European study also looking at breast cancer risk, researchers evaluated two truncating variants in FANCC, p.R185X and p.R548X. They reported the variants in 25/64,760 cases and 26/49,793 controls and therefore neither variant is associated with risk of breast cancer. However, they detected PALB2/FANCD2* p.R414X truncating variant in 22/64,780 cases and 3/49,825 controls, which is associated with breast cancer risk, ductal morphology, early age at diagnosis and low differentiated tumors [70]. Identifying new FA protein variants allows researchers to characterize the protein's function and mechanism, which may differ from previously known roles. In previous studies, we showed FANCD2-V2 is a more potent tumor suppressor than the known form FANCD2, FANCD2-V1. Our lab recently determined the role of FANCD2-V2 in response to early DNA damage. FANCD2-V2 responds earlier to DNA damage, via UVB treatment, than FANCD2-V1. FANCD2-V2 is localized in the nucleus and cytoplasm as compared to FANCD2-V1, which is solely nuclear. We identified a binding partner of FANCD2-V2, Trk-fused gene, TFG, which protects FANCD2-V2 from proteasome degradation.

Interrupting binding or silencing TFG showed less FANCD2 foci formation, increased oncogenicity and sensitivity to UVB and MMC treatment (Ma & Hokutan unpublished). In addition, another group examined the subcellular localization of FANCD2 in patients with ovarian cancer and showed nuclear and cytoplasmic localization was observed in normal and cancer patients. However, those with cytoplasmic FANCD2 have a significantly longer median survival time than patients without cytoplasmic FANCD2 [71], further demonstrating FANCD2-V2 as a more potent tumor suppressor.

Studies of the FA pathway at the protein level have shown FA proteins are overexpressed in cancers, providing researchers with targets for effective treatment strategies. Clinicians can also risk-stratify patients based on FA protein isoforms in cancer, such as breast. However, these associations no longer hold in larger studies. Further studies of FA protein variants and mutants are necessary to determine unknown functions and mechanisms involved in tumorigenesis.

METABOLITE LEVEL

The metabolome is the set of metabolites synthesized by a biological system [72]. Metabolomics investigates these

metabolites in biofluids, cells and tissues [73] and is divided into targeted and untargeted studies. Targeted studies determine the concentrations of pre-defined metabolites, while untargeted studies utilize a global approach [74]. Changes at all preceding levels of biological information flow occur during cancer initiation and progression, which alters cell metabolism. Cancer cells increase anabolic synthesis and adapt to less nutrients and oxygen being available [75]. Alternative pathways used by cells influences metabolites produced and therefore provides the opportunity to identify biomarkers for cancer diagnosis and prognosis and to develop targeted therapy [73-76].

There have been several studies examining the FA pathway at the metabolomic level. Our lab continued studies on the FANCL variant, FAVL and showed cells with an impaired FA pathway exhibit a metabolic signature of tumorigenesis. We used human bladder cancer cells, T24 and produced cells expressing high or low FAVL. We showed eight metabolites (2-oxoglutaric acid, D-pantothenic acid, L-aspartic acid, L-methionine, L-phenylalanine, L-threonine, L-valine and oleic acid) resulting in increased cell proliferation and five metabolites (L-alanine, L-methionine, L-phenylalanine, L-tryptophan and L-threonine), which are end products of inhibiting cell death were elevated in FAVL high vs low cells [77]. Additionally, we studied another FA protein, FANCC and reported FANCC provides protection from metabolic disorders. We compared cells with different levels of FANCC and found alterations in metabolites were associated with aging (glycine, citrulline, ornithine, L-asparagine, L-tyrosine, L-arginine, L-glutamine, L-leucine, L-isoleucine, L-valine, L-proline and L-alanine), diabetes (carbon monoxide, collagens, fatty acids, D-glucose, fumaric acid, 2-oxoglutaric acid, and C3), inflammation (inosine, L-arginine, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, hypoxanthine and L-methionine), and cancer (L-methionine, sphingomyelin, acetyl-L-carnitine, L-aspartic acid, L-glutamic acid, niacinamide, and phosphorylethanolamine) [78].

Another group used lipidomics to study loss of FA genes in HNSCC. They previously showed FA gene loss stimulates HNSCC cells to display mesenchymal morphology and enhanced cell motility and invasion. HNSCC cell invasion was linked to plasma membrane projections, and these phenotypes are dependent on DNA-PK/Rac1 signaling. Next, they used a mass spectrometry (MS)-based lipidomics approach to define FA pathway-dependent lipid metabolism and extract lipid-based signatures and effectors of invasion in FA-deficient cells. They subjected FA-isogenic HNSCC keratinocyte cell lines to untargeted and targeted lipidomics analyses to discover biomarkers and targets in FA-deficient cells. They found an elevation of glycosphingolipids and that ganglioside upregulation is needed for HNSCC cell invasion. NB-DNJ, a glycosphingolipid biosynthesis inhibitor, diminished ganglioside levels and decreased

invasion of FA deficient cancer cells. They identified a lipid signature is associated to FA pathway loss in HNSCC and glycosphingolipid synthesis inhibitors could be used to treat HNSCC in FA or in FA deficient tumors [79].

Metabolomics, the newest of the “omics” disciplines, has provided the field with important information such as identifying novel predictive biomarkers and pinpointing pathways affected by drug therapy. Although, few studies regarding the FA pathway have been done, we see FA pathway loss alters the metabolic and lipid signature in bladder cancer and HNSCC cells, respectively. Continued metabolomics and multi-omics studies will further increase our understanding of FA signaling in cancer.

CONCLUSION AND PERSPECTIVE

The cancer field has gained much information from studying FA signaling. Each biological level has therapeutic potential, such as identifying predictive biomarkers and drug targets. Early diagnosis of cancer is vital and biomarkers can be used to identify these patients. Such examples of biomarkers in diagnosis include DNA mutations, RNA variants, protein isoforms and alterations in metabolites. Our lab identified the FANCD2-V1/FANCD2-V2 mRNA expression ratio is positively associated with tumor stages and grades. The methylation status of a proximal or distal promoter in FANCD2 promotes the production of one FANCD2 variant over the other. The Me-D/Me-P ratio was similarly associated with tumor stages and grades [55]. Both ratios can assist in early cancer diagnosis. Biomarkers can also be used to monitor disease progression. Alternatively, spliced variants, miRNA expression as well as metabolite changes can determine cancer progression.

Studies of the FA pathway have also been beneficial in regards to predicting treatment response. Researchers report BRCA1 promoter hypermethylation predicts PARP inhibitor (PARPi) response in cancers. PARP1 and PARP2 are involved in DNA damage sensing and repair through base excision repair (BER), single strand break repair (SSB) and double strand break (DSB) repair pathways. Chronic lymphocytic leukemia (CLL) cells have significant defects in DSB repair pathways. Methylation of nine promoter regions of DNA repair proteins were examined in 26 CLL primary samples. No changes in BRCA1, BRCA2, FANCC, F, L, ATM, MGMT, hMLH1 and H2AX promoter methylation were detected, except in two cases of minor BRCA1 hypermethylation. However, BRCA1 mRNA expression was reduced in CLL samples compared to non-malignant lymphocytes. CEP-8983, a PARPi, displayed cytotoxicity and in combination with bendamustine, a nitrogen mustard analog approved for CLL treatment, a synergistic effect was seen in the majority of CLL samples [80]. Others examined the response of high-grade serous ovarian carcinoma (HGSOC) to PARPi, rucaparib. They found variable dose-dependent responses in chemo-naïve

BRCA1/2 mutated patient-derived xenografts (PDX) and no response in PDX with without DNA repair pathway defects. Homozygous methylation of BRCA1 predicts rucaparib response, while heterozygous methylation is associated with resistance. They found methylation occurs after chemotherapy and suggest analyzing BRCA1 methylation pre-treatment will identify those patients that will respond to PARPi treatment [81]. Another group examined the effectiveness of the combination of a PARPi, Talazoparib, and a DNA methyltransferase inhibitor (DNMTi), Vidaza or 5-AZA in NSCLC. NSCLC have abnormal DNA methylation patterns and DNMTi can be used as a treatment option. They reported 5-AZA and Talazoparib decreases clonogenicity and exhibits synergistic cytotoxicity. Additionally, H2AX foci formation and PARP1 binding to damaged DNA increases, and RAD51 recruitment decreases. They found DNMTi decreases the expression of DSB repair and HR genes, creating a BRCAness phenotype. And PARPi and DNMTi therapy sensitizes NSCLC cells to ionizing radiation in vitro and in vivo. The same group showed this combination therapy also enhances cytotoxicity in BRCA proficient TNBC, AML, and ovarian cancer [82].

Researchers continue to test combination therapy to overcome drug resistance. Chemotherapy is the standard therapy for TNBC; however, patients rarely improve as drug resistance occurs. Researchers examined if metformin in combination with cisplatin would be an effective treatment to prevent cisplatin resistance in TNBC cells. They report the combination of cisplatin and metformin decreased cell viability, suppressed RAD51 upregulation by decreasing RAD51 protein stability and increased its ubiquitination. Further, they showed RAD51 knockdown enhances cisplatin activity and the combination treatment exhibits a synergistic anticancer effect in vivo [83].

Progressive studies in each aspect of biological research witness intricate information flow, which is certainly not in a linear fashion, rather in a very dynamic manner that is coordinated with a variety of longitudes and latitudes. Each aspect described above is promising to substantially impact human cancer, in terms of understanding cancer pathogenesis, and improving its diagnosis and prognosis. Unfortunately, cancer death remains high. The huge research effort we made seems under-awarded. How we scientists go onward may need to adjust substantially towards more effectively dealing with cancer and other diseases? Recently, system biology emerges to be a field of studies that would be a little closer to nature, but the question is how we can perform/design studies that are spontaneously combining each micro-aspect in connecting with the natural systems. In this way, we would unlikely end up being a blind man who touches an elephant, and our research would obtain the actual impact as anticipated.

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