Promoter Hypermethylation of *FANCF:* Disruption of Fanconi Anemia-BRCA Pathway in Cervical Cancer

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Abstract

Patients with advanced stage invasive cervical cancer (CC) exhibit highly complex genomic alterations and respond poorly to conventional treatment protocols. In our efforts to understand the molecular genetic basis of CC, we examined the role of Fanconi Anemia (FA)-BRCA pathway. Here, we show that *FANCF* gene is disrupted by either promoter hypermethylation and/or deregulated gene expression in a majority of CC. Inhibition of DNA methylation and histone deacetylases induces *FANCF* gene re-expression in CC cell lines. *FANCF*-deregulated CC cell lines also exhibit a chromosomal hypersensitivity phenotype after exposure to an alkylating agent, a characteristic of FA patients. We also show the involvement of *BRCA1* gene by promoter hypermethylation or down-regulated expression in a small subset of CC patients. Thus, we have found inactivation of genes in the FA-BRCA pathway by epigenetic alterations in a high proportion of CC patients, suggesting a major role for this pathway in the development of cervical cancer. Thus, these results have important implications in understanding the molecular basis of CC tumorigenesis and clinical management in designing targeted experimental therapeutic protocols.

Introduction

Approximately 500,000 new cases of cervical cancer (CC) are diagnosed worldwide each year, and the majority of affected women with advanced cancer die (1). Human papillomavirus is considered as a common risk factor for CC. Most women with early-stage tumors can be cured by surgery and radiotherapy. However, treatment of late-stage and recurrent CC remains largely ineffective. The poor prognosis of advanced CC is due to lack of understanding of its biology at the molecular level and of effective treatment regimens. CC cells harbor complex genomic alterations and are highly unstable (2). A better understanding of molecular alterations could therefore facilitate the design of new and targeted therapies for individualized treatment.

Fanconi anemia (FA) is an autosomal recessive chromosomal instability syndrome characterized by hypersensitivity to DNA cross-linking agents and predisposition to cancer, especially leukemia (3). FA patients are also prone to various solid malignancies, including squamous cell carcinoma. The lifetime risk for developing CC in FA patients is also significantly higher than in control populations (3, 4). FA is a genetically heterogeneous disease with genes for seven FA complementation (FANC) groups identified (5). FANC genes are essential in DNA repair pathways in a normal cellular response to cisplatin and other DNA cross-linking agents. FANC proteins interact with BRCA genes in a pathway that involves a number of other genes (6). Recently, it has been shown that promoter hypermethylation of *FANCF* gene disrupts the FA-BRCA pathway, resulting in cisplatin resistance (7). *FANCF* promoter hypermethylation has also shown to occur in squamous cell carcinomas of lung and oral cavity (8).

Because FA patients exhibit an increased risk for the development of CC, we investigated whether the FA-BRCA pathway is altered in this tumor.

Materials and Methods

Patients, Tumor Tissues, and Cell Lines. A total of 100 CC DNA samples derived from 91 at-diagnosis tumor biopsies from invasive CCs and nine cell lines was used in these studies. The tumor biopsies were ascertained from patients evaluated at the Instituto Nacional de Cancerologia (Santa Fe de Bogota, Colombia) and from the Department of Obstetrics and Gynecology of Friedrich Schiller University (Jena, Germany) after appropriate informed consent and approval of protocols by Institutional Review Boards. The primary tumors were clinically classified as International Federation of Gynecologists and Obstetricians stage IB (18 tumors), IIB (23 tumors), IIIB (47 tumors), and IV (3 tumors). Histologically, 86 tumors were classified as squamous cell carcinoma and 5 as adenocarcinoma. Clinical information was collected as described previously (9). Follow-up ranged from 1 to 72 months. Cytologically diagnosed cervical swabs from 18 normal, 11 atypical squamous cells of undetermined significance, 19 low- and 7 high-grade squamous intraepithelial lesions were collected in PBS from patients attending the Gynecologic Oncology Clinic at Columbia University Medical Center (New York, NY), after appropriate informed consent. The CC cell lines HeLa, SiHa, SW756, C-4I, Ca Ski, C-33A, HT-3, MS751, and ME-180 were obtained from the American Type Culture Collection (Manassas, VA) and grown in tissue culture according to the supplier's recommendations. DNA and/or RNA were isolated from frozen tumor tissues or cell pellets by standard methods. RNA was obtained from 20-μm sections with H&E staining of adjacent sections to evaluate for tumor content. Only sections that contained >70% tumor cells were used for RNA preparation.

Methylation-Specific PCR. Genomic DNA was treated with sodium bisulfite as described previously (9). Placental DNA treated *in vitro* with *Sss*I methyltransferase (New England Biolabs, Beverly, MA) and normal lymphocyte DNA treated similarly with sodium bisulfite were used as controls for methylated and unmethylated templates, respectively. The primers for amplification of methylated and unmethylated promoters of *BRCA1*, *BRCA2*, and *FANCF* (two sets covering regions −86 to +97 and +279 to +431 bp) were designed as described previously (9). PCR products were run on 2% agarose gels and visualized after ethidium bromide staining.

Mutation Analysis and Human Papillomavirus Detection. Six sets of primers covering the coding region of *FANCF* gene were designed to amplify genomic sequences up to 250 bp. Single-strand conformation polymorphism analysis was performed on PCR products generated in the presence of α [^{32}P]dCTP and running 6% nondenaturing polyacrylamide gels containing 10% glycerol. Purified PCR products from representative tumors were sequenced to identify mutations. Human papillomavirus types were identified as described earlier (9).

Drug Treatment and Chromosome Preparations. Cells in culture were treated with mitomycin C at a concentration of 20, 40, or 80 ng for 48 h and exposed to Colcemid for the last 2 h. Metaphases were prepared by standard protocols. A total of 100–150 metaphases was analyzed from replicate exper-

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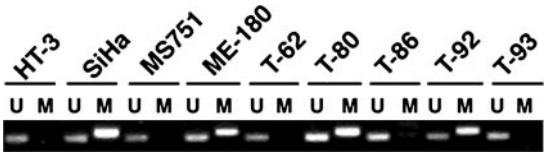

Fig. 1. Methylation-specific PCR analysis of *FANCF* gene in cervical cancer cell lines and primary tumors. U, unmethylated; M, methylated. T, tumor.

iments to identify chromatid- and chromosome-type aberrations. Cell lines were treated with 5-aza-2′deoxycytidine (Aza-C) and trichostatin (TSA) as described previously (9).

Reverse Transcription-PCR Analysis. Total RNA isolated from treated and untreated cell lines, tumor tissues, and three normal cervix uteri obtained from different commercial sources was reverse transcribed as described previously (9). A multiplex semiquantitative analysis of gene expression of *BRCA1*, *BRCA2*, *FANCFA*, *FANCC*, *FANCE*, *FANCF*, and *FANCG* was performed in replicate experiments as described previously (9). A given gene was considered down-regulated in a tumor when the level of mRNA was less than mean-2SD calculated from the expression in normal cervix. All primers used in the present study are available from the authors upon request.

Oligonucleotide Microarray Gene Expression Analysis. Biotinylated cRNA preparation and hybridization to Affymetrix U133A oligonucleotide microarray (Affymetrix, Santa Clara, CA), which contains 33,000 genes, was performed by the standard protocols supplied by the manufacturer. Arrays were subsequently developed and scanned to obtain quantitative gene expression levels. Expression values for the genes were determined using the Affymetrix GENECHIP software and the Global Scaling option, which allows a number of experiments to be normalized to one target intensity to account for the differences in global chip intensity. To perform the supervised gene expression analysis, we used the Genes@Work software platform, which is a gene expression analysis tool based on the pattern discovery algorithm Structural Pattern Localization Analysis by Sequential Histograms (10, 11). The support value for the analysis was chosen as $n = n_o - 2$ (n_o, number of samples in each of the two groups).

Statistical Analysis. Association between promoter hypermethylation and various clinical and genetic characteristics was performed using a χ^2 test.

Results and Discussion

***FANCF* and *BRCA1* Genes Are Hypermethylated in CC.** We previously reported that the 11p15 region to which the *FANCF* gene maps exhibit frequent loss of heterozygosity (LOH) in CC (12, 13). Because the FA-BRCA pathway has been shown to be disrupted by promoter hypermethylation of *FANCF* gene in ovarian, lung, and oral cancers (7, 8), we reasoned that *FANCF* gene may be the target tumor suppressor at 11p15 LOH in CC. To test this hypothesis, we examined the status of *FANCF* promoter hypermethylation in DNA samples from 18 normal cervical epithelia and 100 CC. *FANCF* promoter hypermethylation was not detected in normal cervical epithelium. However, hypermethylation was found in 30% of primary tumors (27 of 91 specimens) as well as in three of nine cell lines (Fig. 1; Table 1). We previously reported the status of 11p LOH in 42 of the primary tumors included in the present study (13). Fourteen of the 42 had an 11p LOH. Promoter hypermethylation of *FANCF* was observed in 5 of the 14 (35.7%) tumors that exhibited 11p LOH compared with 6 of 28 (21.4%) tumors without evidence of 11p LOH. These differences were statistically not significant, suggesting that the changes affecting 11p by LOH and promoter hypermethylation in CC may be independent events.

Correlation of *FANCF* hypermethylation with clinicopathological features showed no relationship with stage, size of the tumor, clinical outcome, and human papillomavirus type. However, patients <45 years showed a significantly higher frequency of promoter methylation compared with the patients >45 years of age (44.4%; 20 of 45 tumors *versus* 15.2%; 7 of 46 tumors; $P = 0.001$). These data, therefore, suggest that *FANCF* promoter hypermethylation plays a role in initiation or progression of CC in younger patients. It has also been previously reported that inherited or somatic mutations in *FANCC* and *FANCG* genes predispose to pancreatic cancer at younger age (14). Taken together, these results suggest that alterations of genes in the FA-BRCA pathway may result in increased risk for the development of cancer in younger age group patients.

Promoter hypermethylation is also known to inactivate other genes in the FA-BRCA pathway, including *BRCA1* and *BRCA2* (15). In the same cohort of cases studied here, we previously reported *BRCA1* promoter hypermethylation in 6.1% of CC patients (9). Promoter hypermethylation of *FANCF* and *BRCA1* genes was mutually exclusive in these tumors. In the present study, we also studied *BRCA2* promoter methylation and found it in none of the tumors. The frequency of *FANCF* promoter hypermethylation seen in the present study is the highest in any tumor reported thus far (7, 8). Thus, in ≥35% of CC patients, either *FANCF* or *BRCA1* was inactivated by promoter hypermethylation, suggesting a major role for the FA-BRCA pathway in this tumor. To identify the role of *FANCF* in CC progression, we studied DNA obtained from 37 pap smears diagnosed at various stages of precancerous lesions by methylation-specific PCR and found no evidence of hypermethylation of the promoter, suggesting that the *FANCF* inactivation is a late event in the tumor development.

FANCF Expression Is Down-Regulated in Most CC Cell Lines. The FA proteins (Fanca, Fancc, Fance, Fancf, and Fancg) that are ubiquitously expressed in dividing normal cells play a major role by forming nuclear complex mediating monoubiquitination of Fancd2 in response to DNA damage (6). To test the role of FA-BRCA pathway genes, *BRCA1*, *BRCA2*, and five FANC genes (*FANCA*, *FANCC*, *FANCE*, *FANCF*, and *FANCG*) were studied for the expression by semiquantitative reverse transcription-PCR analysis in CC cell lines and/or primary tumors. Examination of steady-state levels of *BRCA1* and *BRCA2* genes in nine cell lines and 7 primary tumors, which did not exhibit promoter methylation of these genes, showed no evidence of down-regulated expression of *BRCA2*. However, a relative decrease in

Table 1 *FANCF promoter methylation and RNA expression in cervical cancer cell lines*

Cell line	Promoter hypermethylation	Expression[a]				Mitomycin C sensitivity
		Normal	5-Aza-2′deoxycytidine	Trichostatin	5-Aza-2′deoxycytidine + Trichostatin	
CaSki	No	+	+	+	+	Low
HT-3	No	+	+	+	+	ND[b]
SiHa	Yes	↓	+++	+++	+++	ND
ME-180	Yes	↓	+++	+++	+++	High
SW756	Yes	↓	+++	+	+++	High
MS751	No	↓	+++	+++	+++	ND
C-4I	No	↓	+	++	++	High
HeLa	No	↓	+	++	++	ND
C-33A	No	+	++	++	++	ND

[a] Downward arrow, complete lack of or decreased expression compared to expression in normal cervix; +, expression similar to normal cervix; ++ and +++ indicate increased levels of expression.
[b] ND, not done.

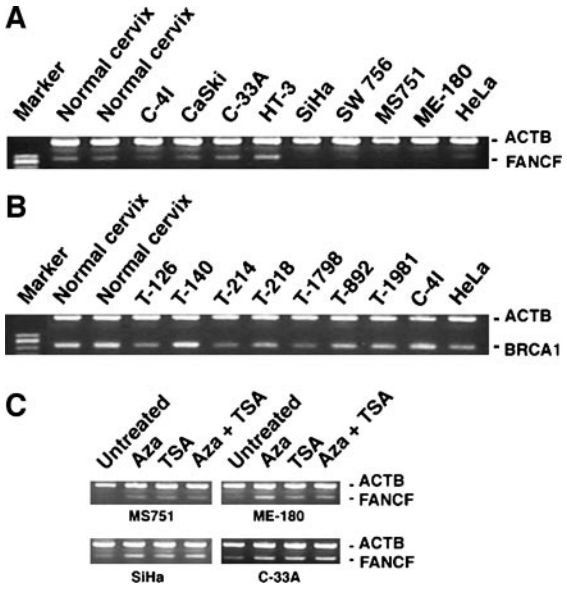

Fig. 2. Semiquantitative analysis of *FANCF* and *BRCA1* expression by reverse transcription-PCR in cervical cancer cell lines and primary tumors. *ACTB*, β-actin; T, primary tumor; *A*, *FANCF* expression in CC. Because *FANCF* coding region spans in a single exon, we treated the total RNA with two rounds of DNase to avoid amplifying contaminating DNA. Note the down-regulated expression of *FANCF* in the cell lines C-4I, SiHa, SW756, MS751, ME-180, and HeLa. *B*, *BRCA1* expression in CC. Decreased levels of *BRCA1* expression was found in T-126, T-214, and T-1798. *C*, effect of demethylation and acetylation on *FANCF* gene expression. Two rounds of DNase treated total RNA was used in reverse transcription reactions to avoid amplifying contaminating DNA.

the expression of *BRCA1* was found in 3 of 7 (43%) primary tumors but not in the cell lines (Fig. 2*B*).

We then examined the levels of mRNA of five FA genes (*FANCA*, *FANCC*, *FANCE*, *FANCF*, and *FANCG*) in nine CC cell lines. The *FANCA*, *FANCC*, *FANCE*, and *FANCG* genes showed no evidence of decreased expression (data not shown). However, the *FANCF* gene showed a down-regulated expression in all three cell lines (SiHa, SW756, and ME-180) that exhibited promoter hypermethylation (Fig. 2*A*). In addition, three other cell lines (C-4I, MS751, and HeLa), which did not show promoter methylation, also showed down-regulated *FANCF* mRNA compared with normal cervix (Fig. 2*A*; Table 1). Overall, six of nine (66.6%) cell lines exhibited *FANCF* gene down-regulation. Thus, the present results suggest that *FANCF* is inactivated by mechanisms other than complete methylation of its promoter such as partial methylation or mutation. To evaluate the latter possibility, we examined mutations in the coding region in a panel of 16 methylated and 16 unmethylated CC. No nonsense or frameship mutations could be found. However, this analysis identified five different sequence variants present both in tumor as well as in the corresponding peripheral blood DNA, four in the coding region and one at the 5′-noncoding region, that were not reported in the single nucleotide polymorphism (SNP) database.[8] These were TCG→TTG at the −10 position, CGC→CGT at codon Arg^{32} without change in amino acid, GCC→GTC that changes Ala^{186}→Val, AAG→AGG that changes Lys^{203}→Arg, and CCT→CTT that changes Pro^{320}→Leu. The Pro^{320}Leu has previously been reported as a normal variant (16). Whether these germ-line variants truly represent polymorphisms or inherited mutations that predispose the patients to cancer remain to be tested.

Because *FANCF* is a component of FA complex and plays a crucial role in DNA damage repair by mediating downstream FANCD2 monoubiquitination, we wanted to examine if gene expression differences exist between tumors carrying hypermethylated and unmethylated *FANCF* promoter (6). Supervised analysis of expression profile of 7 *FANCF* methylated tumors and 8 unmethylated tumors identified a small number of 12 genes differentially expressed between the two groups (Fig. 3). Microarray analysis confirmed *FANCF* expression is associated with promoter hypermethylation (Fig. 3). Nucleoplasmin (*NPM1*) is the only gene that showed higher levels of expression in promoter hypermethylated tumors compared with unmethylated tumors. Eleven genes, most of which are members of immune response family, showed down-regulated expression in *FANCF*-hypermethylated tumors (Fig. 3). Role of these genes in relation to DNA repair pathway is unclear. Therefore, these results suggest that *FANCF* inactivation by promoter hypermethylation is only upstream regulator in DNA damage response, and no consistent quantitative changes in expression of downstream genes occur in normal cellular conditions.

FANCF Is Reactivated in Response to Cellular Exposure to Demethylating Agents. To test whether other epigenetic mechanisms such as partial methylation and histone deacetylation play a role, we examined *FANCF* expression after treatment with Aza-C, TSA, or both. Aza-C or TSA induced reactivation of *FANCF* in all three cell lines (SiHa, SW756, and ME-180) that exhibited complete promoter hypermethylation and down-regulated expression. Two additional cell lines (C-33A and MS751) without evidence of promoter hypermethylation also induced *FANCF* expression in response to exposure to Aza-C or TSA (Fig. 2*C*; Table 1). Two other cell lines (C4-I and HeLa) with unmethylated promoters also induced expression of *FANCF* after exposure to TSA but not with Aza-C. Thus, seven of nine (78%) CC cell lines exhibited deregulated expression by epigenetic mechanisms of inactivation.

DNA hypermethylation-mediated gene silencing is closely associated with histone modifications such as methyl-H3-K9. In this regard, DNA-demethylating agents Aza-C and histone deacetylase inhibitor TSA reactivates expression of epigenetically silenced genes (17). Although DNA hypermethylation is essential to maintain repressive state of histone code, histone modifications precede DNA hypermethylation in silencing specific genes (18, 19). In the present study, reactivation of *FANCF* after exposure to TSA in the absence of promoter methylation suggests that

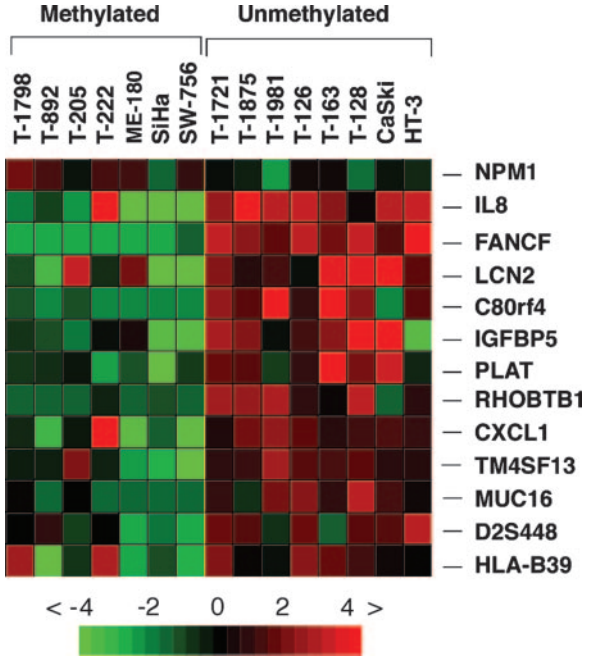

Fig. 3. Supervised analysis of gene expression in FANCF promoter hypermethylated and unmethylated cervical cancer. In the matrix, each row represents a gene and each column represents a sample. The color change in each row represents the gene expression relative to the mean across the samples. The *scale bar* at the bottom represents the difference in the Z_{ge} score (expression difference/SD) relative to the mean.

[8] http://www.ncbi.nlm.nih.gov/SNP/.

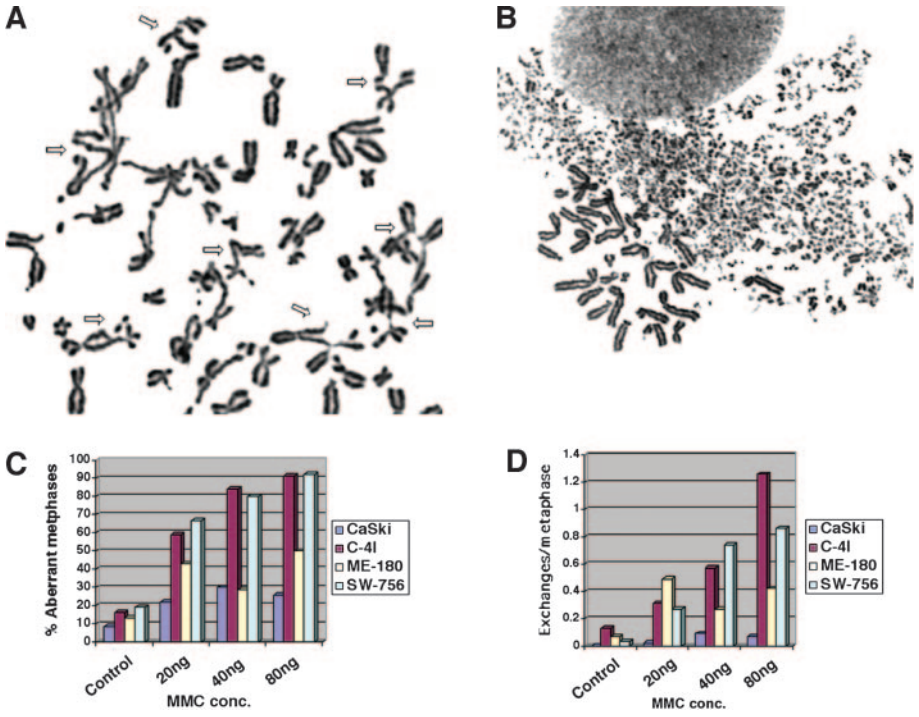

Fig. 4. *FANCF* inactivated cervical cancer cell lines are hypersensitive to mitomycin C (MMC) treatment. *A*, MMC-induced chromosome breakage in SW756 cell line. A representative micrograph showing chromosome radials (*arrows*). *B*, a near tetraploid metaphase from C-4I cell line showing MMC-induced multiple breakages. *C* and *D*, MMC-induced chromosomal aberrations in *FANCF*-inactivated cell lines. Percentage of aberrant metaphases with all types of aberrations (*C*) and multiradials/metaphase (*D*) are shown. The cell line CaSki had no evidence of *FANCF* defect. C-4I cell line showed no evidence of *FANCF* promoter methylation but exhibited down-regulated expression and activation by treatment with TSA. ME-180 and SW756 cell lines had *FANCF* promoter hypermethylation and down-regulated expression.

key histone modifications, either by direct or indirect involvement of promoter methylation, also play a role in down-regulating *FANCF* gene expression in CC.

CC Cell Lines Exhibit Chromosomal Hypersensitivity to Mitomycin C. FA cells exhibit increased chromosome breakage and multiradial formation after exposure to DNA-damaging agents (20). To examine the chromosomal sensitivity in relation to *FANCF* inactivation, we exposed four cell lines (one without evidence of defects in *FANCF* and three with *FANCF* deregulated expression) to various concentrations of mitomycin C. The cell lines ME-180 and SW756 with methylated promoters and the C-4I cell line without a methylated promoter but with down-regulated and reactivated *FANCF* expression upon treatment to TSA showed a several-fold increase in chromosomal breakage, including multiradials compared with CaSki, a cell line that did not exhibit any *FANCF* defect (Fig. 4). Frequency of aberrant metaphases was increased 2–3.5-fold in *FANCF*-defective cell lines (C-4I, ME-180, and SW756) compared with the CaSki cell line (Fig. 4*C*). However, the metaphases exhibiting multiradial chromosomes were 14–25-fold higher in cell lines with *FANCF* epigenetic alteration (Fig. 4*D*). We also found that the cell lines C-4I and SW756 exhibit a high frequency of polyploid metaphases with extensive chromosomal breakage (Fig. 4*B*). Thus, this pronounced mitomycin C-induced chromosomal hypersensitivity exhibited by CC cell lines with *FANCF* epigenetic alterations is similar to the phenotype seen in FA patients.

Our results suggest that epigenetic inactivation of FA-BRCA pathway is common in CC. Tumor cells carrying *FANCF* or *BRCA1* promoter hypermethylation are hypersensitive to DNA-damaging drugs and may result in pronounced tumor death because of their underlying defect in FA-BRCA pathway. It has been shown earlier that progression of ovarian cancer is related to *FANCF* promoter hypermethylation and demethylation of the promoter results in cisplatin resistance (7). Additional studies are required to determine the role of *FANCF* promoter hypermethylation in causing cisplatin resistance in CC. However, identification of this important molecular pathway may have implications in designing appropriate chemotherapy regimen to be used in combination with radiotherapy and a genotype-based therapy may produce a long-term improvement in the treatment of advanced stage CC.

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