

## SHORT REPORTS

**Inactivation of the Fanconi anemia/BRCA pathway in lung and oral cancers: implications for treatment and survival**Carmen J Marsit<sup>1</sup>, Mei Liu<sup>1</sup>, Heather H Nelson<sup>1</sup>, Marshall Posner<sup>2</sup>, Makoto Suzuki<sup>3</sup> and Karl T Kelsey<sup>\*1</sup><sup>1</sup>Department of Cancer Cell Biology, Harvard School of Public Health, Boston, MA 02115, USA; <sup>2</sup>Division of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA 02115, USA; <sup>3</sup>Hamon Center for Therapeutic Oncology, University of Texas Southwestern Medical Center, Dallas, TX, USA

**Inactivation of the FANCF–BRCA pathway via promoter methylation of the FANCF gene renders cells sensitive to DNA crosslinking agents, and has been identified in ovarian cancer cell lines and sporadic primary tumor tissues. We investigated epigenetic alterations in the FANCF–BRCA pathway in head and neck squamous cell carcinomas (HNSCC) and non-small-cell lung cancers (NSCLC) using methylation-specific PCR. Promoter methylation of FANCF occurred in 15% (13/89) of HNSCCs and 14% (22/158) of NSCLCs. Methylation of BRCA1 occurred only in 6/158 NSCLC, and was limited to adenocarcinomas and large-cell carcinomas of the lung. No methylation of BRCA2 was detected. FANCF methylation was associated with a shorter duration of tobacco use ( $P=0.03$ ) and a younger age of starting smoking ( $P=0.06$ ) in NSCLC, and with a greater number of years of alcohol drinking ( $P=0.02$ ) in HNSCC. In adenocarcinomas of the lung, FANCF promoter methylation was a significant predictor of poor survival with a hazard ratio of 3.1 (95% CI 1.2–7.9). This study demonstrates that inactivation of the FANCF–BRCA pathway is relatively common in solid tumors and may be related to tobacco and alcohol exposure and survival of these patients.**

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Fanconi anemia (FA) is an autosomal recessive disease characterized by early onset, progressive aplastic anemia (Joenje and Patel, 2001). Individuals with this disorder have diagnostic cytogenetic sensitivity to the induction of chromatid-type aberrations and chromosome interchanges by DNA crosslinking agents (e.g. diepoxybutane) when their peripheral blood lymphocytes are cultured *in vitro* (Sasaki and Tonomura, 1973; Auerbach and Wolman, 1976; Ishida and Buchwald, 1982). Homozygous carriers of FA are cancer prone,

with a marked predisposition to acute myeloid leukemia (Alter, 1996), and now, after relatively common successful bone marrow transplantation, there is increasing evidence that survivors are at a high risk of solid tumors, especially oral cancers (Kutler *et al.*, 2002).

FA is a genetically heterogeneous disease, comprised of eight ‘complementation groups’, for which all but one of the genes responsible have been identified and cloned (Joenje and Patel, 2001). Together, these genes (along with BRCA1) functioning in the common signaling pathway regulating the cellular response to DNA damage can be considered ‘caretaker’ tumor-suppressor genes, and may therefore be targets of inactivation in familial as well as sporadic cancers (D’Andrea and Grompe, 2003). Until recently, studies investigating epigenetic inactivation of the FANCF–BRCA pathway in solid tumors have focused upon hypermethylation of BRCA1 and BRCA2. Investigators have demonstrated that hypermethylation of the BRCA1 promoter in sporadic breast and ovarian tumors occurs with frequencies ranging from 13 to 24% and 13 to 50%, respectively (Baldwin *et al.*, 2000; Esteller *et al.*, 2000, 2001; Rice *et al.*, 2000; Miyamoto *et al.*, 2002), but methylation of BRCA2 has not been observed (Gras *et al.*, 2001). Beyond breast and ovarian cancers, promoter hypermethylation of BRCA1 was observed in one of 22 (4%) primary lung tumors, but in zero of 18 primary liver cancers, and zero of 18 primary colon cancers (Esteller *et al.*, 2001). More recently, methylation of FANCF in ovarian cell lines and tumors has been associated with loss of protein expression and inactivation of the downstream FA pathway. This inactivation results in these cells demonstrating similar phenotypic sensitivity to DNA crosslinking agents seen in FA patient lymphoblasts. The finding of promoter methylation of FANCF in sporadic ovarian cancers provides an additional mechanism, beyond methylation of BRCA1 and BRCA2 that can lead to the disruption of this pathway. Tumors with this alteration may share the same sensitivity to crosslinking chemotherapeutics, as observed in the methylated cell line or FA patient lymphoblasts, and thus patients with this alteration may benefit from treatment with these drugs (Taniguchi *et al.*, 2003).

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As FA patients exhibit an increased risk for oral cancers, and since identification of alterations in this pathway may provide markers of patients amenable to treatment, we utilized methylation-specific PCR (MSP), using primers specific to both methylated and unmethylated alleles of BRCA1 (Esteller *et al.*, 2000) and BRCA2 (Gras *et al.*, 2001) and FANCF (Taniguchi *et al.*, 2003) in case series studies of NSCLC (Kim *et al.*, 2001b) and HNSCC (Hasegawa *et al.*, 2002) to determine the frequency and impact of inactivation of this pathway on the clinical and epidemiological characteristics of patients (Figure 1). Additionally, to characterize the effects of this alteration *in vitro*, FANCF methylation was also determined in a number of cancer cell lines. Development of many of the cell lines has previously been described (Phelps *et al.* 1996). The remaining lines were obtained from the American Type Culture Collection (Mannassas, VA, USA). Fresh frozen tumor specimens of NSCLC were obtained from 185 volunteer patients diagnosed with lung cancer and undergoing surgical resection at the Massachusetts General Hospital Thoracic Surgery, Oncology, and Pulmonary Services between November 1992 and January 1996. To study HNSCC, fresh frozen tumor specimens were obtained from 94 volunteer patients diagnosed with HNSCC from the Head and Neck Oncology Programs of the Dana Farber Cancer Institute, Brigham & Women's Hospital, or the Beth Israel Deaconess Medical Center, being treated by surgical resection or biopsy, between 1995 and 2000.

The patients involved in these studies provided written informed consent under a protocol approved by the appropriate Institutional Review Boards. Information on smoking history, alcohol consumption, as well as other demographic and clinical characteristics were elicited in questionnaires that were reviewed by a trained interviewer at the time of treatment or were obtained from medical chart review.

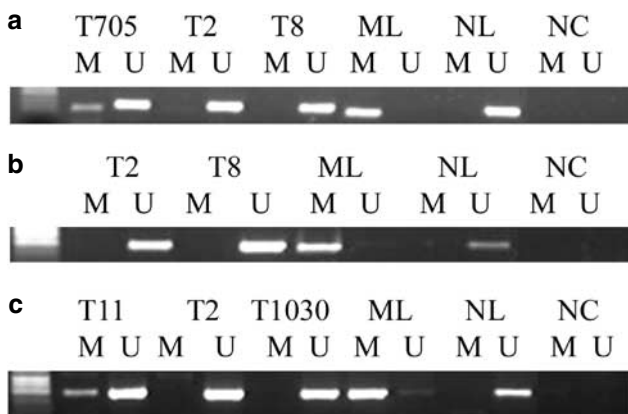
The prevalence of BRCA1 promoter methylation was only 4% (6/158) in NSCLC (Table 1), with 5/84 adenocarcinomas methylated and 1/14 large cell carcinomas methylated. Consistent with the lack of BRCA1 methylation in lung squamous cell carcinoma (0/60), no methylation of the BRCA1 promoter was detected in HNSCC (0/89). BRCA1 methylation was not associated with demographic or clinical factors including age, gender, tumor size, or tumor stage. BRCA1 inactivation was not associated with methylation of the CDKN2A, RASSF1, or DAPK (Kim *et al.*, 2001a, b) loci (data not shown). Consistent with previous studies of BRCA1 methylation in ovarian cancer, methylation of this locus was not associated with TP53 mutation in the adenocarcinomas (Wang *et al.*, 1995), nor was this methylation associated with asbestos exposure, or any quantitative measure of smoking. Methylation of BRCA2 was not detected in any of the samples examined.

Similar to the findings of BRCA1 and BRCA2 hypermethylation in this and previous studies, methylation of FANCF was not found in any of the cancer cell lines tested, including 20 NSCLC lines (H596, H358, H2126, H1734, H1781, H969, H2087, H226, H522, A549, CRL5802, EK VX, HOP62, H322 M, H460, H23, H226, HOP92, CRL5802, H522), 10 SCLC lines (DMS114, SHP77, H69, H187, H1618, H1963, H526, H345, H82, HCC33), three colon cancer lines (HCT116, HCT15, SW480), two bladder cancer lines (UMUC-3, HTB9), an osteosarcoma cell line (SaOS), a breast cancer cell line (MCF7), and a prostate cancer cell line (DU145).

In contrast, methylation of FANCF occurred considerably more frequently than BRCA1/2 inactivation in primary tumor specimens, with 14% of NSCLCs and 15% of HNSCCs having methylated FANCF promoters (Table 1). Promoter inactivation of FANCF was not associated with age, gender, tumor size, histology, or tumor stage in either the HNSCC or NSCLC series.

In HNSCC, methylation of FANCF occurred significantly more frequently in former smokers compared to current smokers (Table 2). Consistent with this finding, there was a 10-year difference in the median number of years smoked by FANCF methylation status in lung SCC cases (45 years for the FANCF unmethylated compared with 35 years for the FANCF methylated tumors). More specifically, the lightest smokers (those smoking for less than or equal to 36 years, the lowest quartile) were almost five times more likely to have FANCF methylation compared to those smoking for greater than 36 years (OR 4.9; 95% CI 1.2–20.6).

Alcohol use, in combination with tobacco smoke, is a significant risk factor for oral cancer (Rothman and



**Figure 1** MSP for BRCA1, BRCA2, and FANCF. Methylation was detected in DNA extracted from fresh-frozen tumor samples using the QIAmp DNA Mini kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's protocol. Sodium bisulfite modification was performed as previously described (Herman *et al.*, 1996), and the resulting DNA was subjected to PCR amplification using primers and conditions for BRCA1 (Esteller *et al.*, 2000), BRCA2 (Gras *et al.*, 2001), and FANCF (Taniguchi *et al.*, 2003), as previously described. Normal circulating blood lymphocyte DNA (NL) was used as a normal unmethylated control, while *Sss*I methylase-treated TK6 cell line DNA (ML) was used as a positive methylated control. No template controls (NC) were also subjected to PCR as contamination controls. The left lane contains *Msp*I-digested pBR322 DNA as a size marker. MSP results for (a) BRCA1, (b) BRCA2, and (c) FANCF in tumor samples (T) or controls using primers specific for methylated (M) or unmethylated (U) alleles

**Table 1** *BRCA1* and *FANCF* methylation status by demographic features of the HNSCC and NSCLC populations<sup>a</sup>

|                                | HNSCC (n = 89)          |                           |                         | NSCLC (n = 158)           |                                      |                           |
|--------------------------------|-------------------------|---------------------------|-------------------------|---------------------------|--------------------------------------|---------------------------|
|                                | <i>FANCF</i> methylated | <i>FANCF</i> unmethylated | <i>FANCF</i> methylated | <i>FANCF</i> unmethylated | <i>BRCA1</i> <sup>b</sup> methylated | <i>BRCA1</i> unmethylated |
| Overall, n (row%)              | 13 (15%)                | 76 (85%)                  | 22 (14%)                | 136 (86%)                 | 6 (4%)                               | 152 (96%)                 |
| Age (mean ± s.d.) <sup>c</sup> | 61.3 ± 13.2             | 58.7 ± 12.9               | 65.8 ± 10.6             | 67.2 ± 10.6               | 72.7 ± 6.6                           | 67.0 ± 10.7               |
| Gender, n (row%)               |                         |                           |                         |                           |                                      |                           |
| Female                         | 5 (22%)                 | 18 (78%)                  | 9 (13%)                 | 60 (87%)                  | 3 (4%)                               | 66 (96%)                  |
| Male                           | 8 (15%)                 | 47 (85%)                  | 13 (15%)                | 76 (85%)                  | 3 (3%)                               | 86 (97%)                  |

<sup>a</sup>Data were analysed using SAS statistical analysis software. <sup>b</sup>*BRCA1* methylation was specific to only adenocarcinomas (5/84) and large-cell carcinomas (1/14) of the lung, and was not detected in any HNSCC tumors. <sup>c</sup>s.d. = standard deviation

**Table 2** Associations of *FANCF* methylation with exposure factors in SCC and adenocarcinomas of the lung and HNSCC

|  | <i>FANCF</i> meth. neg. n (row%) | <i>FANCF</i> meth. Pos. n (row%) | Odds ratio (95% CI) for <i>FANCF</i> meth. |
|--|----------------------------------|----------------------------------|--|
| <i>NSCLC-SCC (n = 57)</i>                                    |                                  |                                  |  |
| <i>Smoking status</i>  |                                  |                                  |  |
| Never smoker   | 0                                | 0                                |  |
| Former smoker  | 26 (81%)                         | 6 (19%)                          | 1.2 (0.3–4.9)                              |
| Current smoker   | 21 (84%)                         | 4 (16%)                          |  |
| <i>Years smoking<sup>a</sup>, quartiles</i>                  |                                  |                                  |  |
| ≤ 36 years   | 9 (60%)                          | 6 (40%)                          | 6.0 (1.4–25.9)                             |
| 37–44 years  | 13 (93%)                         | 1 (7%)                           |  |
| 45–53 years  | 12 (92%)                         | 1 (8%)                           |  |
| ≥ 54 years   | 11 (85%)                         | 2 (15%)                          |  |
| <i>NSCLC-adenocarcinoma (n = 69)</i>                         |                                  |                                  |  |
| <i>Age starting smoking<sup>b</sup></i>                      |                                  |                                  |  |
| ≤ 18 years old   | 30 (81%)                         | 7 (19%)                          | 7.2 (0.8–62.3)                             |
| > 18 years old   | 31 (97%)                         | 1 (3%)                           |  |
| <i>HNSCC (n = 89)</i>  |                                  |                                  |  |
| <i>Smoking status</i>  |                                  |                                  |  |
| Never smoker   | 10 (91%)                         | 1 (9%)                           |  |
| Former smoker <sup>c</sup>                                   | 17 (68%)                         | 8 (32%)                          | 4.4 (1.2–16.9)                             |
| Current smoker   | 38 (90%)                         | 4 (10%)                          |  |
| <i>Years drinking, stratified by case median<sup>d</sup></i> |                                  |                                  |  |
| ≤ 37 years   | 14 (100%)                        | 0                                |  |
| > 37 years   | 8 (62%)                          | 5 (38%)                          | 5.2 (1.4–19.9)                             |

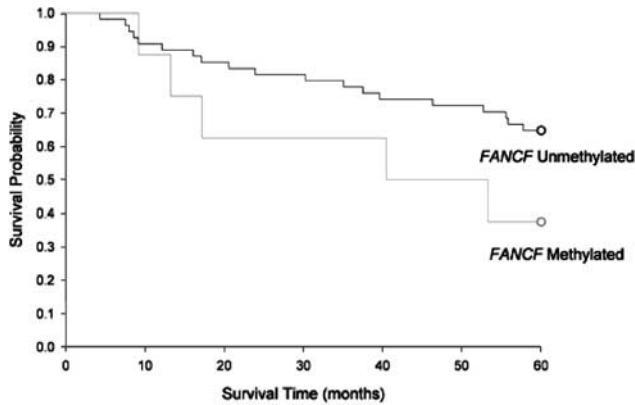
<sup>a</sup>Fisher's exact test for equal proportion of *FANCF* methylation between those in the lowest quartile compared to all other quartiles,  $P = 0.02$ . Data on years smoking were available for 55 cases. <sup>b</sup>Fisher's exact test for equal proportion of *FANCF* methylation between those who started smoking at age 18 years, compared to those who started at > 18 years,  $P = 0.06$ . <sup>c</sup>Fisher's exact test for equal proportion of *FANCF* methylation between former and current smokers,  $P = 0.04$ . Data on smoking status were available for 78 patients. <sup>d</sup>Fisher's exact test for equal proportion of *FANCF* methylation between those drinking for years ≤ case median, compared to those drinking for > case median,  $P = 0.02$ . Data on years drinking were available for 27 cases

Keller, 1972), and (with tobacco exposure) it has been associated with a variety of somatic alterations found in oral cancers (Olshan *et al.*, 1997; Hasegawa *et al.*, 2002). In this study, long-term alcohol use was significantly associated with *FANCF* promoter methylation, as those who drank alcohol for greater than or equal to 37 years were five times more likely to have methylation than those who drank for shorter time periods (OR 5.2; 95% CI 1.4–19.9).

Although no association between the measures of tobacco smoking and *FANCF* methylation was observed in adenocarcinomas of the lung, there was a borderline significant association between methylation

of this gene and a younger age of starting smoking. In this case, the frequency of *FANCF* methylation was greater in those patients who began smoking at age 18 years or younger, compared to those who started later (Table 2).

In examining the effect of *FANCF* methylation on patient outcome, we considered all stage I and II adenocarcinoma patients for whom survival and *FANCF* methylation data were available ( $n = 62$ ). Examining Kaplan–Meier survival probability curves using the log-rank test, there was a borderline statistically significant association between *FANCF* methylation and decreased survival time (Figure 2;  $P = 0.09$ , log-



**Figure 2** Kaplan–Meier 5-year survival probability curves in patients with stage I and 2 adenocarcinoma of the lung, by FANCF methylation status ( $n = 62$ ). Survival time was defined as the time from surgery to the patient’s death, known recurrence, or the last time the patient was known to be alive. Survival probability curves were constructed for various groupings of patients using the Kaplan–Meier method, and differences between groups tested by using the log-rank method

**Table 3** Cox-proportional hazards model of 5-year survival for FANCF methylation in adenocarcinomas limited to stage I and stage II<sup>a</sup> ( $n = 62$ )

|                        | Hazard ratio <sup>b</sup> | 95% CI  |
|------------------------|---------------------------|---------|
| FANCF methylation      | 3.1                       | 1.1–8.8 |
| Stage (ref = stage I)  | 3.8                       | 1.6–9.3 |
| KRAS codon 12 mutation | 1.8                       | 0.7–4.5 |

<sup>a</sup>Cox’s proportional hazards modeling was used to examine the simultaneous effects of several variables on the patient’s outcome. The data were consistent with the assumptions of Cox’s proportional modeling. <sup>b</sup>Controlled for age and gender

rank test). In order to control for the association of additional variables on patient outcome, we performed a multivariate analysis using the Cox’s proportional hazard’s model including, as covariates, FANCF methylation status, stage, gender, age, and KRAS mutation status (Nelson *et al.*, 1999), which this lab has previously found to be associated with poorer survival in these patients (Table 3). Controlling for these covariates, FANCF methylation was a strong, statistically significant predictor of poor outcome in adenocarcinomas, with a hazard ratio of 3.1 (95% CI = 1.2–7.9) (Table 3).

The low prevalence and histologic specificity of BRCA1 methylation and the lack of BRCA2 methylation suggest that inactivation of the FANCF–BRCA pathway via epigenetic silencing of these genes is rare and cell type specific. Silencing of BRCA1 does appear to be selected for and does not occur as a consequence of global hypermethylation, as there were no observed associations between this alteration and methylation of a variety of other tumor suppressors.

The lack of FANCF methylation in the large number of cancer cell lines examined suggests that this alteration is not selected for during *in vitro* growth. It is well

recognized that many lung tumors cannot be propagated *in vitro* and FANCF methylation may be one lesion that contributes to this phenotype. On the other hand, methylation of FANCF occurred at a moderate frequency in NSCLC and HNSCC primary tumors. The lack of associations of this alteration with histology in the NSCLC series was surprising, given that inactivation of BRCA1 occurred only in adenocarcinoma and large-cell carcinoma of the lung. In HNSCC, FANCF methylation occurred more frequently in long-time drinkers, a result that could be related to altered folate uptake. Heavy drinking is associated with low folate levels, which can result in decreases of s-adenosyl methionine (SAM), the major methyl donor in a variety of cellular methylation reactions. Decreases in SAM can lead to global hypomethylation, which has been linked to the induction of a number of cancers (Ehrlich, 2002). Folate deficiency and global hypomethylation may also be related to hypermethylation of tumor-suppressor genes (Potter, 1999; Fang and Xiao, 2001), and thus may be important in defining the susceptible individuals at risk for these epigenetic alterations, including FANCF methylation.

Interestingly, in HNSCC, former smokers were greater than four times more likely to have FANCF methylation than current smokers. In lung SCC, although there was no association between FANCF methylation and smoking status, those who used tobacco for the lowest quartile of years were significantly more likely to be FANCF methylated. There was a positive, although not statistically significant, association of a younger age at starting smoking and methylation of FANCF. Interestingly, if age is included in a multivariate logistic model with these measures of duration, there is no change in the estimate of the effect of duration on FANCF methylation (data not shown), suggesting that these effects are not merely age-associated phenomenon. Taken together, these results demonstrate that shorter smoking duration and intense alcohol consumption are associated with FANCF methylation. These results may indicate that, although this alteration may occur early in the clonal development of the tumor, it is only rarely selected for, and is thus not commonly observed. Cells harboring FANCF methylation may require additional alterations in order to survive and expand.

Inactivation of the FANCF–BRCA pathway has been repeatedly shown to sensitize cells to the action of DNA crosslinking agents such as mitomycin C and cisplatin (Sasaki and Tonomura, 1973; Auerbach and Wolman, 1976; Ishida and Buchwald, 1982). Indeed, ovarian cancer cell lines with methylation silencing of FANCF (indistinguishable from that reported here) are highly susceptible to these treatments, demonstrating dramatic differences in cell survival after *in vitro* exposure (Taniguchi *et al.*, 2003). We have also seen that cancer cell lines, specifically those derived from lung cancer, do not exhibit this alteration, suggesting that this alteration may be detrimental to growth. In our data, patients with FANCF methylation had a three times greater chance of an adverse outcome compared to patients without

FANCF methylation (controlling for age, sex, tumor stage, and KRAS mutation status). These patients may have, beyond the FANCF methylation, additional alterations that allow tumor growth even with this alteration, and it may be these alterations that confer this survival disadvantage. Therefore, in the patients with NSCLC and FANCF promoter methylation, whose survival after surgical treatment is significantly poorer, adjuvant treatment with cisplatin following resection may be highly effective, as the FANCF methylation may still confer a sensitivity to these chemotherapeutic agents. While these tumors may be heterogeneous and contain cells with a malignant phenotype that are not FANCF methylated (and hence not sensitive to cisplatin), treatment trials may be warranted to determine the efficacy of this treatment.

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