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Folic acid supplementation in vitro induces cell type–specific changes in BRCA1 and BRCA 2 mRNA expression, but does not alter DNA methylation of their promoters or DNA repair

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ABSTRACT

Dietary supplementation with folic acid (FA) has been shown to induce opposing effects on cancer-related outcomes. The mechanism underlying such heterogeneity is unclear. We hypothesized that FA supplementation induces changes in breast cancer–associated (BRCA) genes 1 and 2 expression and function through altered epigenetic regulation in a cell type–dependent manner. We investigated the effect of treating normal and cancer cells with physiologically relevant FA concentrations on the mRNA and protein expression, capacity for DNA repair, and DNA methylation of BRCA1 and BRCA2. FA treatment induced dose-related increases in BRCA1 mRNA expression in HepG2, Huh-7D12, Hs578T, and JURKAT and in BRCA2 in HepG2, Hs578T, MCF7, and MDA-MB-157 cells. FA did not affect the corresponding normal cells or on any of the ovarian cell lines. Folic acid induced increased BRCA1 protein expression in Hs578T, but not HepG2 cells, whereas BRCA2 protein levels were undetectable. FA treatment did not alter DNA repair in liver-derived cells, whereas there were transient effects on breast-derived cells. There was no effect of FA treatment on BRCA1 or BRCA2 DNA methylation, although there was some variation in the methylation of specific CpG loci between some cell lines. Overall, these findings show that the effects of FA on BRCA-related outcomes differ between cells lines, but the biological consequences of induced changes in BRCA expression appear to be at most limited.

Abbreviations: ANOVA, analysis of variance; BRCA gene, breast cancer–associated gene; FA, folic acid; PBMC, peripheral blood mononuclear cells.

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1. Introduction

Folic acid (FA) is the synthetic form of folate that is used widely as a nutritional supplement or in dietary fortification. The effect of FA on cancer risk is unclear, and there are conflicting reports that suggest that FA intake is either associated with increased or decreased risk of cancer, in particular colorectal cancer [1]. FA fortification has been associated with a lower incidence of neuroblastoma, but had no effect on lymphoblastic leukemia or hepatoblastoma [2]. Maternal FA intake has been associated negatively with risk of childhood neuroectodermal tumors [3] and neuroblastoma [4]. In adults, supplementation with 5 mg FA per day reduced reoccurrence of adenomas by 56% [5] compared with placebo, whereas cosupplementation of FA and aspirin had no significant effect on reoccurrence [6,7]. The extent to which such effects are associative rather than causal is unclear [8]. Furthermore, the incidence of colorectal cancer in the United States and Canada appeared to increase transiently after the introduction of mandatory FA fortification [9]. This positive association between FA and risk of colon cancer is supported by an increase in incidence by 2.6 to 2.9 between pre- and postintroduction of FA fortification in Chile [10]. In contrast, FA intake was negatively associated with colorectal cancer risk in a case-cohort study of 5629 women [11]. A meta-analysis of randomized controlled trials of FA supplementation based on 13 studies failed to show a significant effect on total cancer incidence or the incidence of specific cancers [12]. Women who received a supplement containing FA and vitamins B12 and B6 showed reduction in risk of total invasive cancer and of breast cancer, although these effects were not statistically significant [13]. Although such heterogeneity may reflect differences between study cohorts and between the design of the intervention, and level of FA given, it is also possible that different tissues or cancer subtypes may differ in their response to FA.

Tetrahydrofolate is the biologically active metabolite of FA and is a cofactor for the rate-limiting reaction in the supply of methyl groups to the homocysteine/methionine remethylation cycle in which DNA is a terminal acceptor. Epigenetic regulation of transcription by DNA methylation involves differential methylation of CpG dinucleotides in gene promoters as well as covalent modifications of histones and noncoding RNAs [14]. Methylation of gene promoters is a relatively stable epigenetic mark that is induced during development. However, some genes retain epigenetic plasticity beyond early development and are susceptible to interventions in later life, including FA intake [15]. Furthermore, aging is associated with carcinogenesis with both global hypomethylation and hypermethylation of tumor suppressor genes [16]. Diets low or enriched in FA have been shown to induce altered DNA methylation in experimental models [17–20] and in humans [21]. Thus, variations in folate status or FA intake may modify cancer risk by altering the epigenetic regulation of genes.

The breast cancer–associated (BRCA) genes 1 and 2 are tumor suppressor genes with several key functions related to maintaining DNA integrity [22]. The proteins encoded by these genes are expressed in all cells and are critical for repair of single- and double-stranded DNA breaks. Mutations in the BRCA1 and BRCA2 genes have been implicated primarily in the development of breast and ovarian cancers, but germline mutation carriers of BRCA1 and BRCA2 also have a small increased risk of stomach, pancreas, prostate, and colon cancer [23]. Impaired BRCA1 and BRCA2 activities lead to gross chromosomal rearrangements and gene dysregulation [22]. Approximately 90% of cases of breast and ovarian cancer are sporadic and are not associated with mutations in the BRCA genes [24]. In these cases, reduced BRCA1 activity involves hypermethylation of its promoter leading to transcriptional repression [25–29]. In contrast, the BRCA2 promoter has been shown to be hypomethylated and overexpressed in ovarian cancers compared with normal tissue [29]. Thus, one possible additional source of heterogeneity in the effects of FA on cancer risk is the differential effects on the epigenetic regulation of BRCA1 and BRCA2 leading to genomic instability [30–32]. In order to inform nutritional guidelines about FA intake and cancer risk, it is important to know if FA supplementation induces differential effects on the epigenetic regulation of BRCA1 and BRCA2, and whether such effects are specific to individual tissues or cancer subtypes and if such effects differ between cancer and normal cells.

We tested the hypothesis that treatment with FA induces differential effects of the epigenetic regulation of BRCA1 and BRCA2 transcription leading to variation between cell types in capacity for DNA repair. To address this, normal and cancer cells were treated in vitro with concentrations of FA that were within the range of unmetabolized FA in plasma (0-100 nmol/L) [33–37] reported in humans taking 200 μg/day FA or more on the mRNA expression of BRCA1 and BRCA2. Cells arising from different tissues were tested to determine whether any effects of FA on BRCA1 and BRCA2 were specific to a specific cancer type or subtype. To determine whether any changes in BRCA1 or BRCA2 mRNA expression were associated altered function of these genes, we investigated the effect of FA treatment on BRCA1 and BRCA2 protein expression and on the DNA methylation of their promoters, and on capacity of cells to repair radiation-induced DNA damage.

2. Methods and materials

2.1. Cell lines

SK-HEP-1 human liver adenocarcinoma, PLC/PRF/5 human liver hepatoma, Huh-7D12 human hepatocellular carcinoma, HMT-3522 S1 human breast epithelia, Hs578T human breast adenocarcinoma, MDA-MB-157 human breast medulla carcinoma, MDA-MB-231 human breast adenocarcinoma, A2780 human ovarian carcinoma, COV434 human ovarian granulosa tumor, and PEA1 human ovarian carcinoma were obtained from the European Collection of Cell Cultures. MCF10a human nontumorigenic breast epithelia were obtained from American Type Culture Collection, peripheral blood mononuclear cells (PBMC) were obtained from Stem Cell Technologies, and primary hepatocytes were obtained from Life Technologies. HepG2 human hepatocellular carcinoma, MCF7 human breast adenocarcinoma, THP1 human acute monocytic leukemia, and JURKAT human acute T cell leukemia cells were from our

archive, which was derived originally from cells purchased from European Collection of Cell Cultures.

2.2. Cell culture procedures

All cell lines were cultured at 37°C in an atmosphere containing 5% (v/v) CO_2, in Dulbecco's modified Eagle medium without FA (Sigma), supplemented with 10% (v/v) fetal bovine serum, 2 mmol/L glutamine, 10 U/mL penicillin, and 100 μg/mL streptomycin. The medium for the MCF10a cell line was further supplemented with 20 ng/mL epidermal growth factor and 100 μg/mL hydrocortisone. The medium for the HMT-3522 S1 cell line was also supplemented with 10 ng/mL epidermal growth factor and 500 ng/mL hydrocortisone.

2.3. Measurement of BRCA1 and BRCA2 mRNA expression by real-time reverse transcription polymerase chain reaction

To determine the effect of FA supplementation on BRCA1 and BRCA2 mRNA expression, all cell lines were treated with 0, 25, 50, 75, or 100 nmol/L FA for 72 hours before harvesting in TRI Reagent (Sigma) according to the manufacturer's instructions. Background folate concentration derived from fetal bovine serum was 1.5 nmol/L. Measurement of mRNA expression was carried out essentially as described previously [38]. Briefly, complementary DNA was prepared using Moloney-murine leukemia virus reverse transcriptase (Promega). Real-time reverse transcription polymerase chain reaction was performed with SYBR Green JumpStart Taq ReadyMix (Sigma) to amplify BRCA1 and BRCA2 mRNA using QuantiTect Primer assays (Qiagen) QT00039305 and QT00008449, respectively. mRNA levels were determined by the standard curve method [39] and normalized to cyclophilin expression (QuantiTect assay QT01866137) [38]. All samples were analyzed in duplicate.

2.4. Measurement of BRCA1 and BRCA2 protein expression by western blotting

BRCA1 and BRCA2 protein levels were assessed in cell lines in which FA treatment induced significant changes in BRCA1 and/or BRCA2 mRNA expression. Cells were treated with either 0 or 100 nmol/L FA. Protein extracts were prepared in 50 mM tris pH 8, 150 mM NaCl, 0.5% sodium deoxycholate, and 1% nonidet-P40 containing 10% (v/v) Protease Inhibitor Cocktail (Sigma). Protein concentrations were determined using a Pierce BCA Protein Assay Kit (Thermo Scientific). Western blot analysis of protein expression was performed as described previously [40]. Cell extract (25 μg) was resolved by SDS PAGE using a 4% to 15% polyacrylamide gradient gel (Bio-Rad) and transferred to PVDF membrane (Amersham) in 25 mM tris pH 8, 192 mM glycine, 20% (v/v) methanol, and 0.1% (w/v) SDS for 3 hours at 4°C. The membrane was blocked with 5% (w/v) skimmed milk powder/tris-buffered saline (TBS; 10 mM tris pH 8.0, 150 mM NaCl) containing 0.1% (v/v) Tween 20 for 1 hour at room temperature and then incubated overnight at 4°C with anti-BRCA1 antibody (1 μg/mL; Abcam) or anti-BRCA2 antibody (2 μg/mL; Abcam) in 2% (w/v) skimmed milk powder/TBS/0.1% Tween 20. The membrane was then washed four times for 10 minutes each in TBS/0.1% Tween 20 before being incubated with a horseradish peroxidise–conjugated anti-mouse secondary antibody (1:50 000; Sigma) in 2% (w/v) skimmed milk powder/TBS/0.1% Tween 20 for 1 hour at room temperature. After washing in TBS/0.1% Tween 20, the protein bands were detected using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and were visualized on a VersaDoc 4000MP imaging system (Bio-Rad). Protein molecular weights were determined using a Fermentas Spectra Multicolor Broad Range protein ladder (Fisher Scientific), and protein band intensities were analyzed using ImageJ software (NIH). Anti–β-actin (1:2000; Sigma) was used as the primary antibody to normalize for differences in protein loading.

2.5. Measurement of BRCA1 and BRCA2 promoter methylation by sodium bisulphite pyrosequencing

The regions of the BRCA1 and BRCA2 genes that were analyzed for DNA methylation by pyrosequencing are indicated in Fig. 1. The region of the BRCA1 promoter that was analyzed has been shown to be unmethylated in normal cells and hypermethylated in cancer [43], to be involved in the regulation of transcription [44] and to contain the minimal promoter [41] and several transcription factor binding sites [45–47]. The region of the BRCA 2 gene that was analyzed has previously been shown to be hypermethylated in sporadic breast cancers [48]. This region encompasses the BRCA2 minimal promoter region [42] and contains a number of transcription factor binding sites that regulate BRCA2 expression [42,49,50].

The level of methylation of individual CpG dinucleotides in the BRCA1 (Fig. 1A) and BRCA2 (Fig. 1B) promoters was measured using bisulphite pyrosequencing essentially as described previously [38]. Genomic DNA was isolated, and bisulphite conversion was performed using the EZ DNA Methylation-Gold kit (ZymoResearch). The bisulphite-modified DNA was then amplified using the primers listed in Table 1 with KAPA2G Robust HotStart ReadyMix (Labtech). Biotinylation of the polymerase chain reaction products allowed them to be immobilized on streptavidin-sepharose beads (GE Healthcare), washed and denatured, and then released into annealing buffer containing the sequencing primers in Table 1. Pyrosequencing was performed using PyroMark Gold Q96 CDT reagents (Qiagen) on a PSQ 96MA machine (Biotage), and the percentage methylation for each CpG loci was calculated using the PyroQ CpG software (Biotage). Internal controls were included within each pyrosequencing assay to verify bisulphite-conversion efficiency. Human genomic DNA methylated at 100% of CpG loci (Millipore) or at 0% CpG loci (Promega) were included for each assay.

2.6. Measurement of DNA repair

Cells were treated with either 0 or 100 nmol/L FA for 72 hours before irradiation with UVC ($\lambda = 254$ nm) at a dose of 0.1 $J/m^{-2}/s^{-1}$ for 18 seconds (1.8 J/m^2) using a CL-1000 UV X-linker (UVP). Cells were cultured for a further 0, 1, or 4 hours and then collected in Ca^{2+} and Mg^{2+}-free PBS at approximately 10^5 cells/mL. Cell viability was determined using trypan blue exclusion (in all experiments viability was ≥90%). The single cell gel

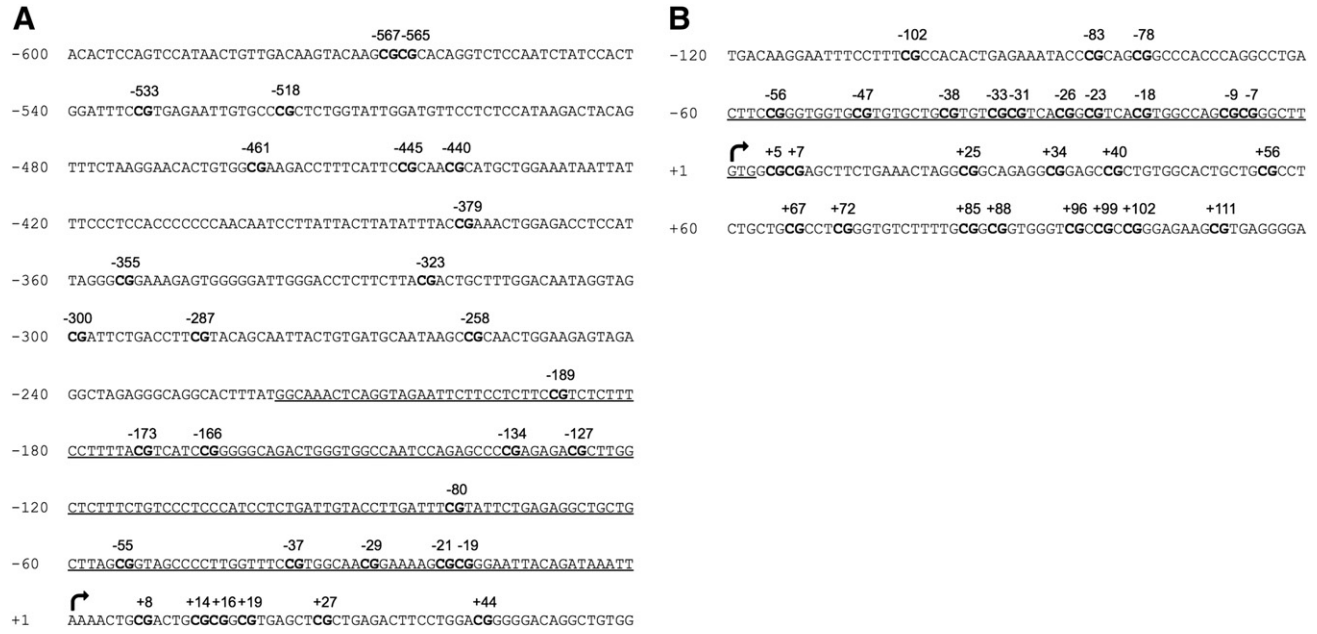

Fig. 1 – Regions of the (A) BRCA1 and (B) BRCA2 genes that were analyzed by pyrosequencing. The minimal promoter regions of BRCA1 [41] and BRCA2 [42] are indicated by the underlined sequences. CpG loci are indicated in bold font and numbered relative to the transcription start site. Arrows indicates the transcription start sites.

electrophoresis assay [51] was performed under alkaline conditions using a Comet Assay Kit (Trevigen). All steps were performed in low light level conditions and at 4°C, unless otherwise stated. Cells were combined with molten low melting point agarose at 37°C at a ratio of 1:10 (v/v), 50 μL was spread onto a CometSlide and the agarose was left to adhere for 30 minutes. The slides were immersed in cold lysis solution overnight and then in freshly prepared, cold alkaline solution (300 nM NaOH, 1 mM EDTA, pH 13) for 1 hour. Slides were then placed in a horizontal electrophoresis tank on ice in alkaline solution and electrophoresis was performed at 15 V (1 V/cm), 300 mA for 1 hour. The slides were washed twice in distilled water and then in 70% ethanol, before being dried for 20 min at 37°C. SYBR Gold (Life Technologies) was used to stain the DNA for 30 min at RT, and the slides were then rinsed with distilled water before being completely dried at 37°C. Comets were imaged using a Nikon D3100 DSLR camera attached to an Axiovert 25CFL microscope (Zeiss). For each treatment, at least 50 cells were analyzed using CASP software (CaspLab), and the amount of DNA damage was expressed as the percentage of total DNA in the comet tail.

2.7. *Statistical analyses*

Data are expressed as means ± SE. Statistical analyses were carried out using SPSS (v21, IBM Corporation, Armonk, NY). FA dose-response groups for each cell line were compared by 1-way analysis of variance (ANOVA) with Dunnett post hoc test. Pairwise comparisons of protein expression and DNA methylation were by Student unpaired *t* test. DNA repair capacity was compared by 2-way ANOVA with Bonferroni post hoc test. Differences were considered to be statistically significant at $P < 0.05$. For the primary outcome measure, mRNA expression, a sample size of 10 cultures provided statistical power of at least 85% for detecting a 10% difference with a two-tailed probability of <0.05. This sample size provided at least this level of statistical power for the other outcomes.

Table 1 – PCR and pyrosequencing primers

CpGs covered	Forward primer	Reverse primer (biotin labeled)	Sequencing primer
BRCA1			
−567 to −518	ATGGGAATTGTAGTTTTTTTAAAGAGTT	AAAAATCCCAATCCCCCACT	AGTTTATAATTGTTGATAAGTA
−355 to −300	AGATTATAGTTTTTAAGGAATATTGTGG	TAAAATACCTACCCTCTAACCTCTACT	ATTGGAGATTTTTATTAGGG
−189 to −166	AGGTTAGAGGGTAGGTATTTTAT	ACTCTAAATTAACCACCCAATCTAC	ATGGTAAATTTAGGTAGAATTTTT
−80 to −19	GGGGTAGATTGGGTGGTTAATTTAGAG	CCAATTATCTAAAAAACCCCACAACC	TTATTTTTTGATTGTATTTTGATTT
+8 to +44	GGGGTAGATTGGGTGGTTAATTTAGAG	CCAATTATCTAAAAAACCCCACAAC	GGGAATTATAGATAAATTAAAATTG
BRCA2			
−56 to +7	GTTGGGATGTTTGATAAGGAATTTT	CACAAATCTATCCCCTCAC	GGT TTATTTAGG TTTGATTT
+25 to +102	GTTGGGATGTTTGATAAGGAATTTT	CACAAATCTATCCCCTCAC	GAGTTT TTG AAATTAGG

3. Results

3.1. *Effect of FA supplementation on BRCA1 and BRCA2 mRNA expression*

FA treatment of liver cancer cell lines induced cell type– and cell line–specific effects on BRCA1 and BRCA2 expression. FA treatment induced a significant increase in BRCA1 and BRCA2 mRNA expression in the hepatocellular carcinoma cell line HepG2 (Tables 2 and 3). FA treatment induced a dose-related increase in BRCA2 expression in hepatocellular PLC/PRF/5 cells, but did not alter BRCA1 mRNA expression significantly. In contrast, in FA-treated hepatocellular carcinoma Huh-7D12 cells, BRCA1 mRNA expression was lower and BRCA2 expression was unchanged. There was no significant effect of FA treatment on BRCA1 or 2 mRNA levels in the liver adenocarcinoma SK-HEP-1 cells or primary hepatocytes (Tables 2 and 3).

There was no significant effect of FA treatment on BRCA1 or 2 mRNA expression in transformed mammary epithelial HMT-3522 cells nor on BRCA1 mRNA expression in the immortalized but nontransformed mammary epithelial MCF10a cells. BRCA2 expression in MCF10a cells was consistently below the detection limit of the assay (Tables 2 and 3). FA treatment increased BRCA1 and BRCA2 mRNA expression in breast adenocarcinoma MCF7 cells. Treatment with FA did not alter BRCA1 mRNA expression significantly in breast medullary MDA-MB-157 cells, but decreased the expression of BRCA2 in a dose-related manner. In contrast, FA treatment induced increased BRCA1 and BRCA2 expression at 25 nmol/L, but the expression of these genes was reduced at higher FA concentrations.

There was no significant effect of FA treatment on BRCA1 expression in any of the ovarian cancer cell lines tested (Table 2), whereas BRCA2 expression was below the assay detection limit (Table 3). Treatment with FA decreased BRCA1 mRNA expression in JURKAT cells, but did not significantly alter its expression in primary PBMC or THP1 cells (Table 2). There was no significant effect of FA treatment on BRCA2 mRNA expression in PBMC, whereas the level of BRCA2 in THP1 and JURKAT cells was below the detection limit of the assay (Table 3).

3.2. *Effect of FA supplementation on BRCA 1 and 2 protein expression*

BRCA1 protein expression was not significantly altered in HepG2 cells exposed to 100 nmol/L FA (Fig. 2A). In contrast, FA treatment of Hs578T cells induced a significant increase in BRCA1 protein (Fig. 2B). The level of BRCA2 protein was below the level of detection in all cells tested (data not shown).

3.3. *Effect of FA supplementation on DNA repair*

Significant DNA damage was induced in all of the liver cell lines which were tested (all $P < .0001$). Treatment with 100 nmol/L FA had no effect on DNA damage in any of the cell lines at any of the time points that were measured (Fig. 3). DNA damage increased significantly in primary hepatocytes 1 hour after being irradiated and the amount of damage returned to similar levels prior to

Table 2 – Effect of FA treatment on BRCA1 mRNA expression

Cell line	Cell type	Relative mRNA expression					ANOVA (P)
		FA concentration (nmol/L)					
		0	25	50	75	100	
Liver							
Hepatocytes	Primary	100 ± 22.4	ND	ND	ND	98.1 ± 18.9	.95[a]
SK-HEP-1	Adenocarcinoma	100 ± 6.2	91.6 ± 6.3	90.3 ± 5.3	93.9 ± 6.4	108.1 ± 8.3	.32
PLC/PRF/5	Hepatoma	100 ± 4.9	99.6 ± 3.1	96.9 ± 5.1	105.6 ± 2.6	107.5 ± 5.9	.45
HepG2	Hepatocellular carcinoma	100 ± 2.4	114.6 ± 2.6**	116.8 ± 3.1**	113.5 ± 1.7*	115.8 ± 4.3**	.003
Huh-7D12	Hepatocellular carcinoma	100 ± 4.1	95.5 ± 4.5	79.0 ± 5.4*	82.0 ± 7.2	98.9 ± 5.0	.024
Breast							
HMT-3522	Transformed epithelial	100 ± 35.8	139.6 ± 36.0	143.9 ± 24.0	174.5 ± 32.1	62.9 ± 58.5	.30
Hs578T	Ductal carcinoma	100 ± 17.0	146.1 ± 11.8	218.1 ± 21.6***	181.5 ± 14.3*	180.5 ± 22.2*	.001
MCF7	Adenocarcinoma (ER+)	100 ± 7.9	92.3 ± 10.0	99.6 ± 13.4	66.8 ± 13.3	119.7 ± 10.5	.028
MDA-MB-157	Medulla carcinoma	100 ± 1.4	121.9 ± 8.0	112.2 ± 6.6	108.5 ± 6.2	98.0 ± 6.4	.080
MDA-MB-231	Adenocarcinoma	100 ± 7.5	124.3 ± 12.8	84.0 ± 4.6	72.4 ± 9.0	77.0 ± 5.0	.0004
MCF10a	Nontumorigenic epithelial	100 ± 20.8	88.7 ± 24.7	ND	64.1 ± 41.1	61.8 ± 27.5	.55
Ovarian							
A2780	Serous carcinoma	100 ± 31.5	163.8 ± 66.0	103.1 ± 32.2	171.4 ± 29.7	131.0 ± 26.4	.52
COV434	Granulosa carcinoma	100 ± 72.8	89.8 ± 62.3	135.7 ± 52.7	141.1 ± 28.6	139.8 ± 33.5	.91
PEA1	Serous carcinoma (ER+)	100 ± 31.6	61.2 ± 21.6	27.8 ± 8.4	43.1 ± 26.0	95.0 ± 29.2	.19
Leukocyte							
PBMC	Primary	100 ± 19.7	97.0 ± 16.3	153.2 ± 23.6	128.7 ± 13.4	119.7 ± 14.2	.197
THP1	Acute monocytic leukemia	100 ± 31.4	150.5 ± 52.8	22.2 ± 7.7	103.6 ± 43.3	169.7 ± 89.7	.0827
JURKAT	Acute T cell leukemia	100 ± 18.2	14.3 ± 12.2*	79.9 ± 36.5	26.8 ± 6.1*	24.8 ± 5.4	.0167

Values are means ± SE (n = 10 replicate cultures). Expression levels were normalized to reference gene and are relative to untreated cells. Data were analysed by 1-way ANOVA using Dunnett post hoc correction except [a]where data were analyzed using Student *t* test. Values significantly different from untreated cells are indicated by *$P < .05$, **$P < .01$, ***$P < .001$.

Table 3 – Effect of FA treatment on BRCA2 mRNA expression

Cell line	Cell type	Relative mRNA expression					ANOVA (P)[a]
		FA concentration (nmol/L)					
		0	25	50	75	100	
Liver							
Hepatocytes	Primary	100 ± 11.9	ND	ND	ND	89.9 ± 17.5	.64[a]
SK-HEP-1	Adenocarcinoma	100 ± 3.5	109.1 ± 13.3	92.8 ± 5.9	113.3 ± 3.0	126.3 ± 13.8	.20
PLC/PRF/5	Hepatocellular carcinoma	100 ± 7.0	100.6 ± 5.4	105.7 ± 5.7	119.2 ± 1.8*	129.2 ± 3.2**	.0009
HepG2	Hepatocellular carcinoma	100 ± 4.1	114.2 ± 2.3	112.0 ± 3.7	111.7 ± 3.8	110.2 ± 5.2	.003
Huh-7D12	Hepatocellular carcinoma	100 ± 6.4	101.1 ± 5.6	88.2 ± 3.2	87.2 ± 3.4	94.6 ± 2.3	.14
Breast							
HMT-3522	Transformed breast epithelial	100 ± 40.7	148.9 ± 55.1	221.7 ± 85.4	278.1 ± 111.9	120.4 ± 120.4	.51
Hs578T	Ductal breast carcinoma	100 ± 18.1	182.9 ± 21.9	334.5 ± 24.9***	228.5 ± 19.1	246.4 ± 26.3*	.0001
MCF7	Breast adenocarcinoma (ER+)	100 ± 10.8	106.0 ± 7.8	123.2 ± 12.6	108.2 ± 8.4	158.0 ± 11.3***	.0024
MDA-MB-157	Medulla Breast carcinoma	100 ± 4.4	86.6 ± 4.4	72.7 ± 4.6	71.4 ± 4.9	62.2 ± 2.0**	.0029
MDA-MB-231	Breast adenocarcinoma	100 ± 9.6	127.9 ± 14.0	110.7 ± 9.8	74.6 ± 10.9	105.6 ± 8.8	.023
MCF10a	Immortalized breast epithelial	ND	ND	ND	ND	ND	ND
Ovarian							
A2780	Serous carcinoma	Undetectable					ND
COV434	Granulosa carcinoma	Undetectable					ND
PEA1	Serous carcinoma (ER+)	Undetectable					ND
Leukocyte							
PBMC	Primary	100 ± 23.4	142.4 ± 15.8	214.2 ± 41.8	235.3 ± 25.6	179.5 ± 18.9	.0672
THP1	Acute monocytic leukemia	Undetectable					ND
JURKAT	Acute T cell leukemia	Undetectable					ND

Values are means ± SE (n = 10 replicate cultures). Expression levels were normalized to reference gene and are relative to untreated cells. Data were analysed by 1-way ANOVA using Dunnett post hoc correction except [a]where data were analysed using Student *t* test. Values significantly different from untreated cells are indicated by *P < .05, **P < .01, ***P < .001.

irradiation (Fig. 3A). HepG2 cells had much lower levels of DNA damage, which were highest immediately after irradiation and then decreased to baseline damage levels after 4 hours (Fig. 3B). Conversely, the damage observed in the PLC/PRF/5 cell line significantly increased with every time point (Fig. 3C).

Significant DNA damage was also induced in all of the breast lines that were tested (all $P < .0001$) (Fig. 4). There was a significant time × treatment interaction effect on DNA damage in MCF10a cells ($F_{3,907} = 12.0$, $P < .0001$) (Fig. 4A). Treatment with 100 nmol/L FA decreased the amount of

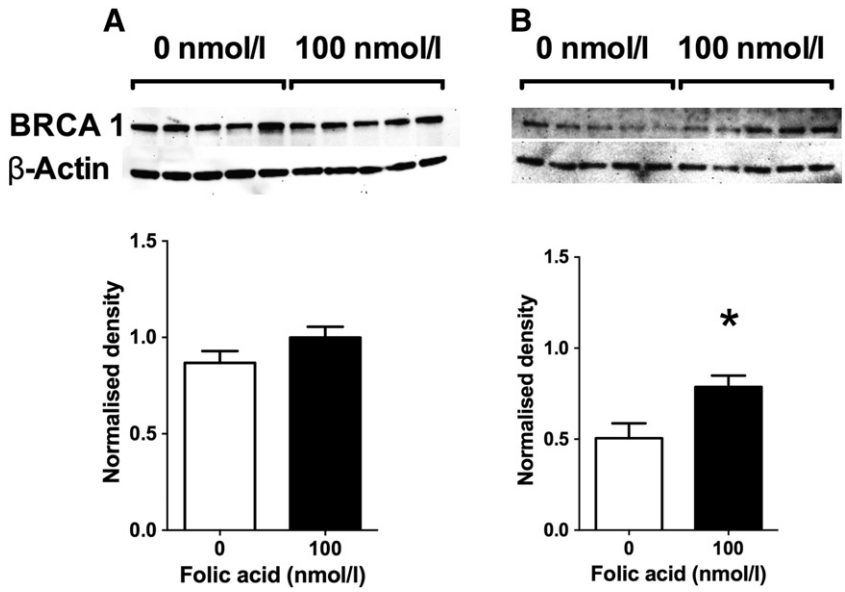

Fig. 2 – Effect of FA treatment on BRCA1 protein expression in HepG2 and Hs578T cells. Cell extracts from (A) HepG2 and (B) Hs578T cells treated with 0 nmol/L FA or 100 nmol/L FA for 72 hours and analyzed by Western blotting with anti-BRCA1 and anti–β-actin antibodies. Values are means ± SE (n = 5 replicate cultures). Data were analyzed using Student *t* test. Values significantly different from untreated cells are indicated by *P < 0.05.

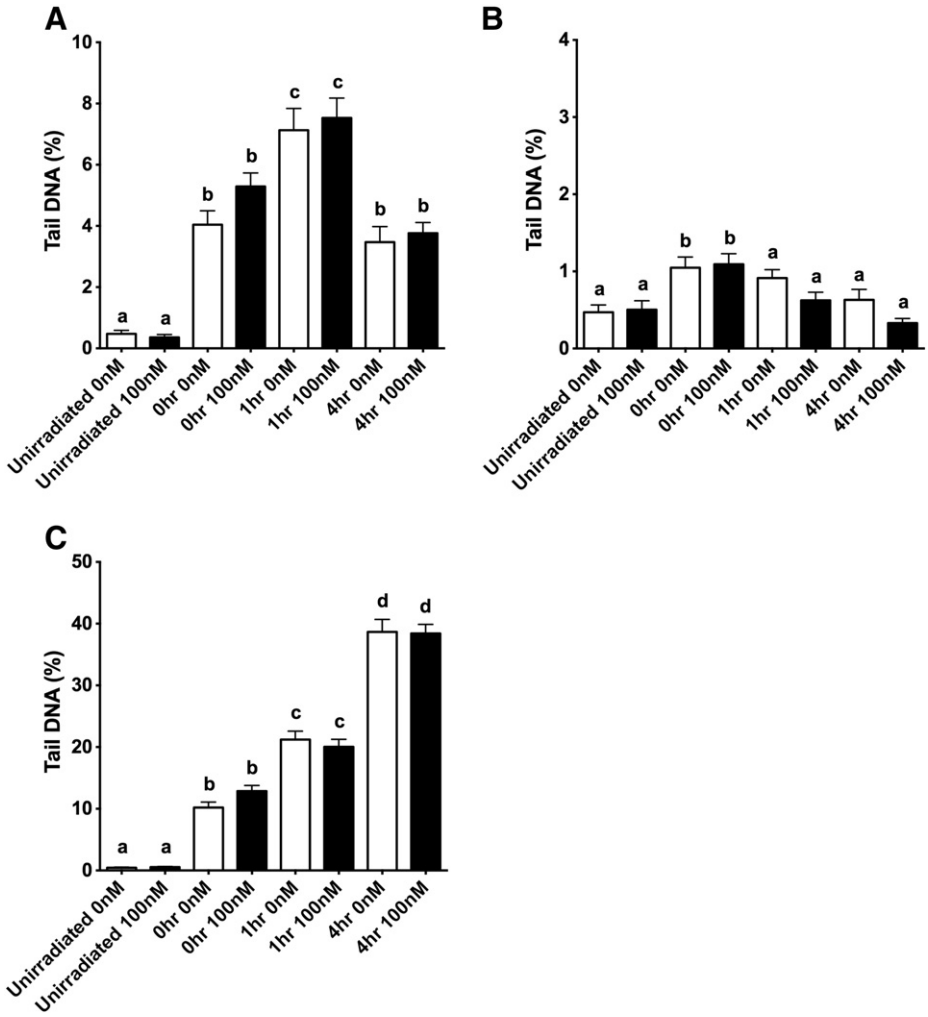

Fig. 3 – Effect of FA supplementation on DNA repair capacity in liver cells. Primary hepatocyte (A), HepG2 (B), and PLC/PRF/5 (C) cells were treated with 0 nmol/L FA or 100 nmol/L FA for 72 hours, irradiated with 1.8 J/m^2 UVC, and DNA damage was analyzed by Comet assay. Values are means ± SE (n ≥ 50 comets). Data were analyzed by 1-way ANOVA using Bonferroni post hoc correction. Means without a common letter differ significantly ($P < 0.05$).

damage observed in MCF10a cells after 1 hour; however, the damage in both treatment groups had returned to baseline levels after 4 hours (Fig. 4A). There was also a significant time × treatment interaction effect on DNA damage in Hs578T cells ($F_{3,911} = 7.2$, $P < .0001$) (Fig. 4B). DNA damage immediately after irradiation was significantly higher in cells treated with 100 nmol/L FA compared to untreated cells. However, DNA damage levels were significantly lower in the FA treated cells than the control group after 1 hour recovery (Fig. 4B). After 4 hours, DNA damage levels for both groups had increased to similar levels. There was no significant effect of FA treatment on the induction of DNA damage or recovery in either MCF7 or MDA-MB-157 cells (Fig. 4C, D).

3.4. *BRCA 1 and 2 DNA methylation*

We compared baseline methylation levels at 0 nmol/L FA for all of the cell lines (Figs. 5 and 6). Because of the detection limit of pyrosequencing assays [52], CpG loci that had methylation levels of 5% or less were regarded as essentially unmethylated. Statistical analysis was only carried out for loci at which the level of methylation was at least 5% in all the cell lines tested for a specific tissue.

BRCA1 promoter methylation was below 15% at most CpGs investigated in all liver cell lines, with small significant differences (≤5%) between cell lines at specific CpG loci (Fig. 5A). Methylation of BRCA1 in the breast cancer cells was more variable than in liver or ovarian cells, or leukocytes (Fig. 5). HMT-3522 and Hs578T cells significantly higher methylation (≥20%) at CpG loci –567, –565 and in HMT-3522 cells alone at CpGs –533 and –518 compared with the other breast cell lines for which methylated was approximately 5% for all CpG loci (Fig. 5B). There were also small, significant differences (≤5%) between ovarian cells lines in the level of methylation at CpGs –533 and –518. Methylation of CpGs –567 and –565 in PBMCs and THP1 cells was significantly higher (20%-30%) at CpGs –567, –565 and at CpGs –533 and –518 (≥10%) compared to the JURKAT cells (Fig. 5D). However, the level of methylation for all other CpG loci was close to or less than 5% for all 3 leukocyte cell lines, which were tested. DNA methylation across the BRCA2

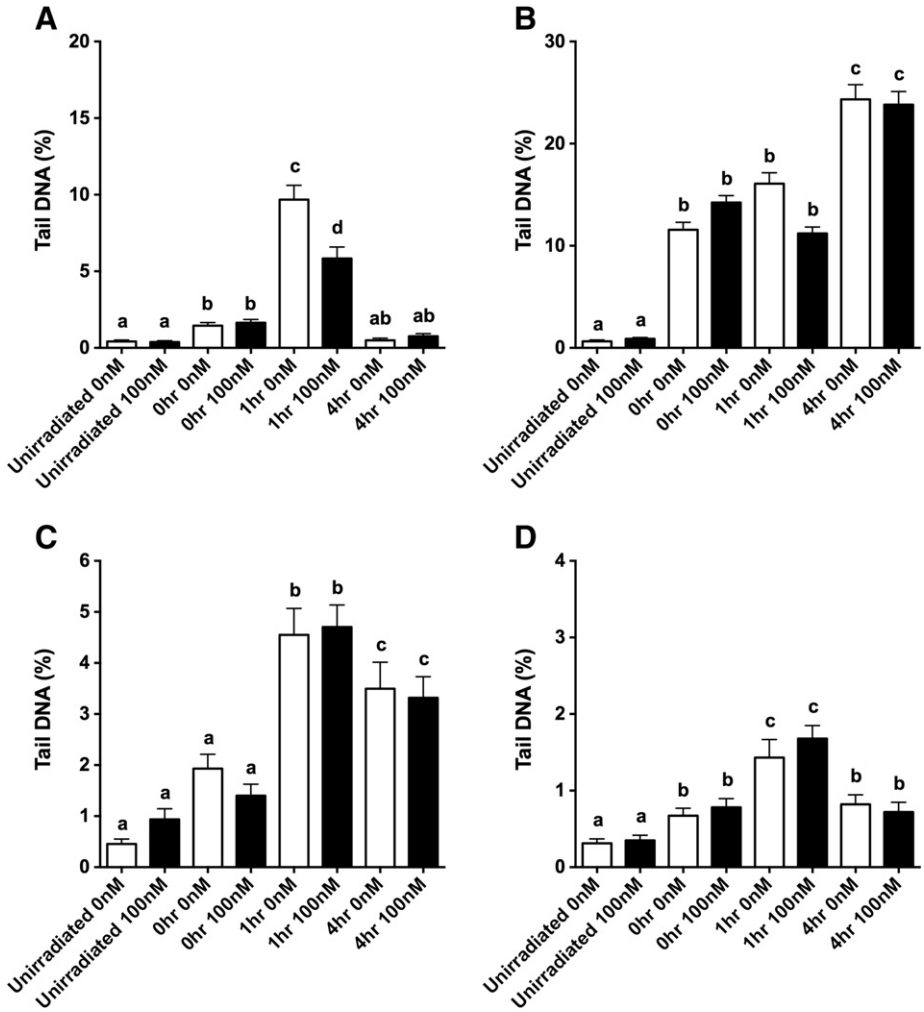

Fig. 4 – Effect of FA supplementation on DNA repair capacity in breast cells. MCF10a (A), Hs578T (B), MCF7 (C), and MDA-MB-157 cells were treated with 0 nmol/L FA or 100 nmol/L FA for 72 hours, irradiated with 1.8 J/m^2 UVC, and DNA damage was analyzed by Comet assay. Values are means ± SE (n ≥ 50 comets). Data were analyzed by 1-way ANOVA using Bonferroni post hoc correction. Means without a common letter differ significantly ($P < 0.05$).

promoter region was close to or below 5% in all of the cell lines investigated (Fig. 6). There was no significant effect of FA treatment on the methylation status of either BRCA1 or BRCA2 in any of the cell lines tested (data not shown).

4. Discussion

The findings of previous studies have suggested that the effect of dietary supplementation with FA on cancer risk is variable and may depend, in part, upon the nature of the cancer [2–5,7,8,10–12,53,54]. Our findings are consistent with these observations. Treatment of cell cultures with FA at concentrations that were within the range which can be achieved in human subjects in vivo [34,55–57] induced differential changes in the mRNA and protein expression of BRCA1 and BRCA2 between primary and cancer cells derived from the same tissue, and between cell lines derived from the same cell type. These findings show for the first time that physiological concentrations of FA are able to modulate the level of mRNA of two genes that encode proteins that are critical for maintenance of DNA integrity. None of the primary or nontransformed cells showed significant FA-induced changes in BRCA1 or BRCA2 mRNA expression. In contrast, 2 of 4 of the liver cancer cells lines, 3 of 5 breast cancer cells lines, and 1 of 2 leukemia cells lines, but none of the ovarian cancer cell lines, showed altered BRCA1 mRNA expression. 2 of 4 liver and 2 of 5 breast, but not ovarian or leukemia, cancer cell lines showed altered BRCA 2 mRNA expression. Although these findings do not represent a comprehensive analysis of all possible cancer cell types that may be derived from these tissues, these findings support the suggestion that any effect of FA supplementation on the mRNA expression of BRCA1 or BRCA2 may reflect the particular type of cancer. Thus these findings are consistent with and suggest an explanation for the inconsistent reports in the literature regarding the effect of FA on cancer risk.

4.1. *mRNA expression*

Treatment with the highest concentration of FA (100 nmol/L) induced changes in the level of BRCA1 protein in the same

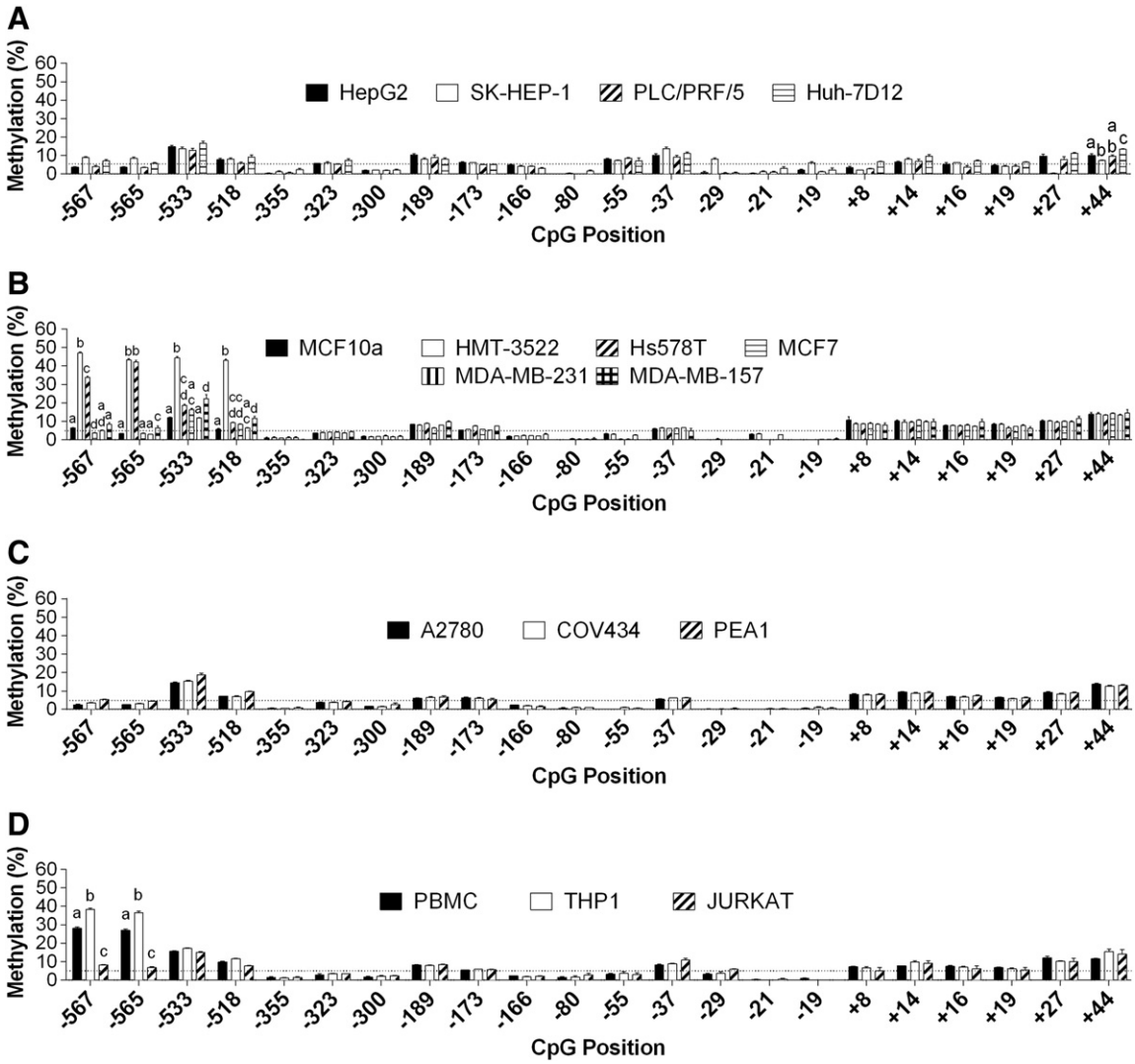

Fig. 5 – BRCA1 DNA methylation. The methylation status of individual CpG loci was measured in liver (A), breast (B), ovarian (C), and leukocyte (D) cell lines without the addition of FA by bisulphite pyrosequencing. Values are means ± SE (n = 10 replicate cultures). Data were analyzed by 1-way ANOVA using Bonferroni post hoc correction. For each CpG loci, means without a common letter differ significantly ($P < 0.05$) (only differences that were ≥5% methylation are marked). Dotted line indicates the limit of detection of the analysis.

direction as the mRNA transcript in HepG2 and Hs578T cells, although this was only significant for the Hs578T cell line. The effect of varying FA concentration on protein expression was not tested for practical reasons. Although MCF7 and MDA-MB-231 cells showed an overall significant effect of FA treatment on BRCA 1 mRNA expression, pairwise testing did not detect a significant difference between treated cells and controls, and so the effect of FA on the levels of BRCA1 protein was not determined in these cells. Although the BRCA2 transcript was detected in some cell lines, the level of BRCA2 protein expression was below the detection limit of the western blot assay. Nevertheless, these findings suggested that, at least in some cell types, FA treatment modified the level of both BRCA1 mRNA and protein. These findings are in contrast to the effect of supraphysiological FA concentrations on normal cells [58]. This highlights the importance of using physiological concentrations in studies of the effects of nutrients on cancer-related outcomes in vitro.

4.2. *DNA repair*

Capacity to repair radiation-induced DNA damage was used to test whether the changes induced in BRCA1 and/or BRCA2 mRNA or protein expression might be biologically significant. All cell types showed significant DNA damage as a result of exposure to nonionizing radiation. However, there were differences between cells types in their ability to repair DNA damage. Primary hepatocytes, HepG2, MCF10a, MCF7, and MDAMB157 cells exhibited DNA repair by 4 hours after irradiation, the extent of which was greater for the noncancer cells hepatocytes and MCF10a cells. However, the other cancer cell lines, PLCPRF5 and Hs578T cells, showed

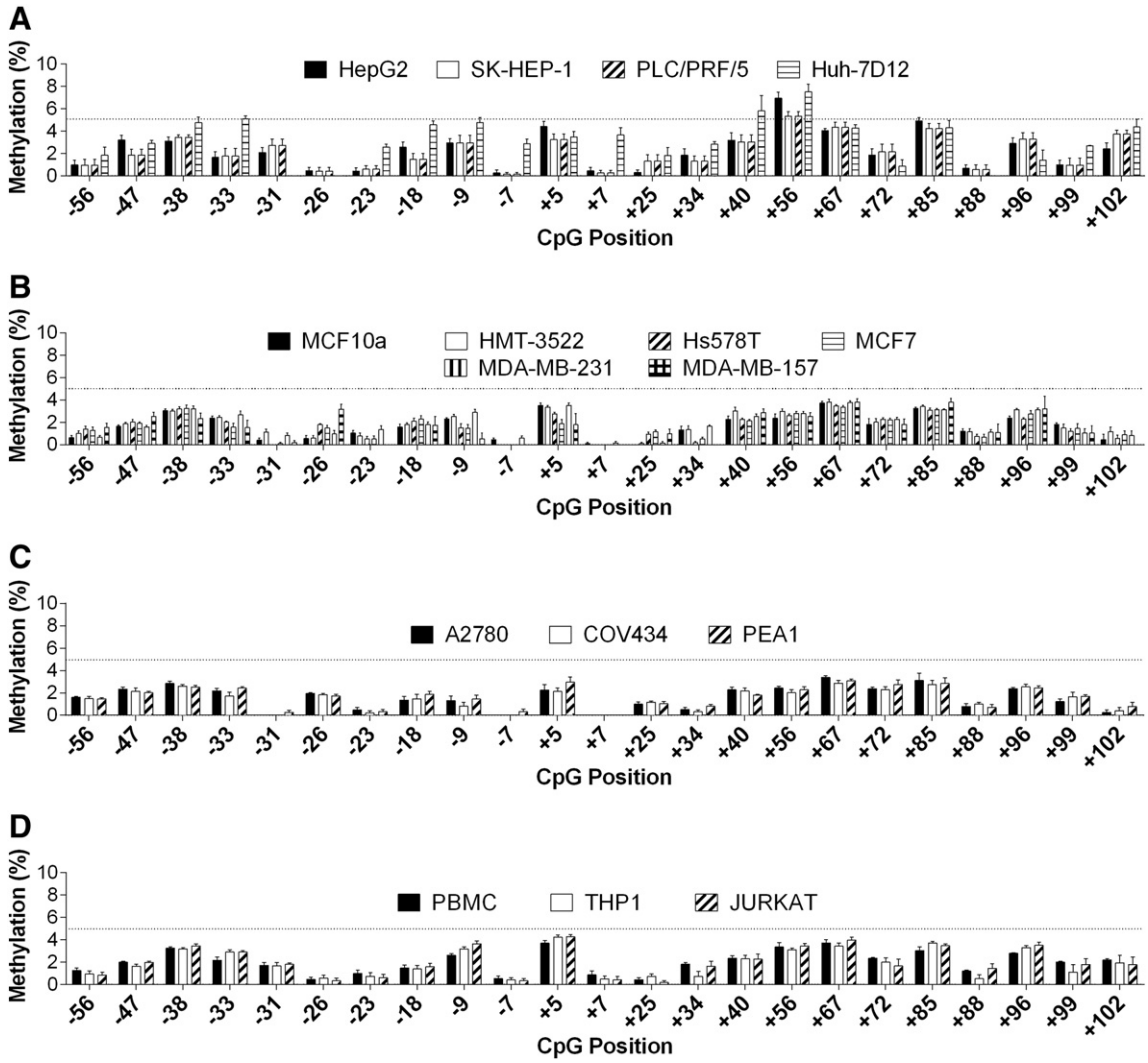

Fig. 6 – BRCA2 DNA methylation. The methylation status of individual CpG loci was measured in liver (A), breast (B), ovarian (C), and leukocyte (D) cell lines without the addition of FA by bisulphite pyrosequencing. Values are means ± SE (n = 10 replicate cultures). Data were analyzed by 1-way ANOVA using Bonferroni post hoc correction. For each CpG loci, means without a common letter differ significantly ($P < .05$) (only differences that were ≥5% methylation are marked). Dotted line indicates the limit of detection of the analysis.

significantly greater DNA damage at 4 hours after irradiation than at earlier time points. Such differences in DNA repair capacity between cell lines may reflect variation in the expression and functional activity of other genes involved in DNA repair. For example, p53 is mutated in Hs578T cells [59] and CDKN2A in PLC/PRF/5 cells [60]. There was no effect of FA treatment on DNA repair in liver-derived cells, whereas there were transient effects of FA treatment on breast tissue-derived cells. One possible explanation is that although FA treatment altered BRCA1 or BRCA2 mRNA expression, the magnitude of this effect maybe too small to result in a significance change in DNA repair capacity. In cancer cells, this may have been due to impaired expression of other genes involved in DNA repair. One implication of these findings is that dietary FA may have a limited effect on the susceptibility of liver or breast tumour cells to radiation and hence may not be a consideration in patients undergoing radiotherapy.

4.3. *DNA methylation*

Variations in folate status have been associated with changes in the DNA methylation status of specific genes [17–20]. Furthermore, DNA hypermethylation of the BRCA1 promoter has been associated with decreased mRNA expression [26,61,62] and with sporadic breast cancer [28,30,63,64]. We investigated whether the changes in BRCA1 or BRCA2 mRNA expression induced by FA treatment were associated with altered DNA methylation of these genes. The region of BRCA1 that was analyzed has been shown previously to be hypermethylated in some sporadic breast cancer cells, but essentially unmethylated in others including MCF7 cells, and in PBMC, fibroblasts and normal mammary epithelium [62]. To our knowledge, there have not been any study that have reported in detail the methylation status of individual CpG loci in BRCA 2 using sequencing techniques. One study reported average

methylation (60%) at CpGs –176 and –148 bp relative to the transcription start site (TSS) [65], but no information is available about the level of methylation of CpG loci more proximal to the TSS. We found that the proximal promoter region of BRCA 1 was essentially unmethylated in all cells tested in the absence of FA treatment. However, specific CpG loci were more highly methylated in some, but not all, breast, ovary and leukocyte-derived cells. In contrast, the region of BRCA2 that was analyzed was essentially unmethylated in all cells tested. One possible implication of these findings is that the background level of DNA methylation, particularly of BRCA1, may influence the choice of cell type for studies on epigenetic processes in cancer.

There was no significant effect of FA treatment on the methylation of the regions of sequenced within the BRCA 1 or 2 promoters. Thus, any effect of FA treatment on the levels of the transcripts of these genes is unlikely to be mediated through changes in DNA methylation of these sequences, although it is possible that other regions could be involved. However, since the duration of FA treatment was relatively short, other mechanisms such as changes in histone methylation could be involved which may subsequently lead to altered DNA methylation over a longer period [66].

5. Conclusions

These findings are consistent with the uncertainty in the literature regarding the effects of FA on cancer risk, but indicate that any effect of FA on BRCA1 or BRCA2 expression may be specific to a particular cell type. Furthermore, the functional consequences of FA appear to be modest at least in terms of DNA repair. Extrapolation of the findings *of in vitro* studies to patients must be cautious and limited. However, one possible implication is that, even if replicated in primary tumour cells, it may not be possible to make general recommendations for FA intake in cancer.

Competing interests

The authors have declared that no competing interests exist.

Author contributions

Conceived and designed the experiments: GCB, KAL. Performed the experiments: RJP. Analyzed the data: GCB RJP. Wrote the paper: GCB, RJP, KAL.

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